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Assessing exposure risk of arsenic, cadmium and lead (or mixed with PAHs) in soils using in-vitro methods

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Abstract

There are over 150,000 contaminated sites in Australia. In most cases, soils are polluted with mixtures of contaminants, including metal/metalloids. However, current human health assessment, particularly in relation to health investigation level guidelines is based on single contaminant. If no site-specific data are available, additive effect is assumed which may overestimate or underestimate the risk. Therefore, a more detailed study on the mixture of metal/metalloids is needed. The emphasis of this study is laid on the exposure risk of arsenic (As), cadmium (Cd), and lead (Pb) when they are ingested as mixtures. Two *in-vitro* models are adopted in this study. Model 1 is the Unified BARGE (Bioaccessibility Research Group of Europe) method (which is known as UBM) with the aim to measure the soluble fraction of soil contaminants in the human gastrointestinal tract (bioaccessibility) as well as the interaction effects of As, Cd and Pb on respective bioaccessibility. Model 2 is using human liver carcinoma cell line (HepG2) to study the subsequent uptake of contaminants into cells in target organ and possible interaction of As, Cd and Pb on their respective uptake after they are solubilised in the human digestive system. Considering at some contaminated sites polycyclic aromatic hydrocarbons (PAHs) may co-exist with As, Cd and Pb, effects of four selected PAHs (NAP for naphthalene, PHE for phenanthrene, PYR for pyrene, and B[a]P for benzo[a]pyrene) on the bioaccessibility and subsequent uptake of As, Cd and Pb into HepG2 cells are also preliminarily investigated. The focus of this PhD study is on mixtures of As, Cd and Pb.

Seven chemically-variant top soils (0-20 cm) were collected in Australia. Soils were characterised for pH, total carbon, total nitrogen, total sulphur, total organic carbon, total inorganic carbon, cation exchange capacity, oxalate-extractable iron, aluminium and manganese, particle size distribution, electrical conductivity etc. A range of concentrations of As, Cd and Pb was spiked into these seven types of soils and aged for up to 12 months. UBM results show the bioaccessibility of As in the gastric phase was not significantly different from those in the intestinal phase whilst a pronounced difference was observed between Cd gastric bioaccessibility and intestinal bioaccessibility as well as between Pb gastric bioaccessibility and intestinal bioaccessibility. Organic carbon, iron oxide and aluminium oxide were key parameters influencing the bioaccessibility of As (gastric and intestinal phases), Cd (intestinal phase) and Pb (intestinal phase).

For mixture studies, two exposure scenarios were investigated. Under the first scenario, As, Cd and Pb were spiked to individual soils to mimic independent ageing of mixed

contaminants, such as contaminants age at different spots or there has been a long time gap between different contaminants entering the same spot. The interaction between binary mixtures of As, Cd and Pb in simulated human digestive system was studied by mixing soils spiked with As, Cd or Pb of the same type in the same extraction container. No interaction effects of As, Cd and Pb on their respective bioaccessibility were observed in any of the seven soil types. Under the second scenario, As, Cd and Pb were spiked into the same soil sample and aged concurrently. Results show As and Pb would interfere the sorption of Cd in soils, leading to a temporary increase in Cd intestinal bioaccessibility. No effects of PAHs (PYR/B[a]P) on the bioaccessibility of As, Cd and Pb were observed in both exposure situations.

UBM-extracted As, Cd and Pb, which represent the available fractions for further uptake after digestive system, were administered to HepG2 cells in order to elucidate the uptake of As, Cd and Pb at hepatic level so as to study whether they would interfere with each other's uptake during this absorption process. Data to date indicate that the uptake of Cd was inhibited in the presence of As and Pb while As and Pb stayed unaffected by Cd. For cells dosed with binary mixtures of metals/metalloid (As/Cd/Pb) and PAHs (PYR/B[a]P), interaction was only detected between Cd and PYR/B[a]P during the accumulation in hepatocytes. These results did not conflict with previous *in vivo* data, which suggests HepG2 cells might be useful *in vitro* model to study accumulation interaction at hepatic level.

The present study is the first one to explore possible interactions among As, Cd and Pb (or mixed with PAHs) in simulated human digestive system as well as interactions during the accumulation in target organ (e.g. liver) cells. Under most environmental conditions, additive effect can be assumed in the digestive system for mixtures of As, Cd and Pb (or mixed with PAHs) whilst subsequent uptake at hepatic level was less than additive for Cd mixed with As/Pb or PAHs. Taken together, interaction should be interpreted individually for different physiological processes. This study provides significant implications for the understanding of interaction among As, Cd and Pb after ingestion and helps to refine the current risk assessment of mixed contaminants.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Xia, Q., Peng, C., Lamb, D., Kader, M., Mallavarapu, M., Naidu, R., Ng, J.C., 2016b. Effects of arsenic and cadmium on bioaccessibility of lead in spiked soils assessed by Unified BARGE Method. Chemosphere 154, 343-349.

Xia, Q., Peng, C., Lamb, D., Mallavarapu, M., Naidu, R., Ng, J.C., 2016a. Bioaccessibility of arsenic and cadmium assessed for in vitro bioaccessibility in spiked soils and their interaction during the Unified BARGE Method (UBM) extraction. Chemosphere 147, 444-450.

Sun, H.Y., **Xia, Q**., Liu, R.T., 2014. Comparison of the binding of the dyes Sudan II and Sudan IV to bovine hemoglobin. J Lumin 148, 143-15.

Peng, C., Muthusamy, S., **Xia, Q**., Lal, V., Denison, M.S., Ng, J.C., 2015. Micronucleus formation by single and mixed heavy metals/loids and PAH compounds in HepG2 cells. Mutagenesis 30, 593-602.

Peng, C., Arthur, D.M., Sichani, H.T., **Xia, Q.**, Ng, J.C., 2013. Assessing benzene-induced toxicity on wild type Euglena gracilis Z and its mutant strain SMZ. Chemosphere 93, 2381-2389.

Conference abstract

Xia, Q., Peng, C., Ng, J.C., 2014, Interaction between cadmium and arsenic when using the united BARGE method(UBM) to measure bioaccessibility, Proceedings of Conference, 7th Annual Scientific Meeting, Sydney, Australia.

Xia, Q., Peng, C., Ng, J.C., 2015. Bioaccessibility of arsenic, cadmium and lead in spiked soils and possible interaction during bioaccessibility extraction, Proceedings of Conference, the 8th International Workshop on Contaminant Bioavailability in the Terrestrial Environment, Nanjing, China.

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Contributor	Statement of contribution
Qing Xia	Designed experiments (80%) Carried out experiments (80%) Data analysis (100%) Wrote and edited the paper (70%)
Cheng Peng	Edited the paper (5%)
Dane Lamb	Edited the paper (5%) Carried out experiments (20%)
Megharaj Mallavarapu	Edited the paper (5%)
Ravi Naidu	Edited the paper (5%)
Jack C. Ng	Designed experiments (20%) Edited the paper (10%)

Incorporated in Chapter 3:

Xia, Q., Peng, C., Lamb, D., Kader, M., Mallavarapu, M., Naidu, R., Ng, J.C., 2016b. Effects of arsenic and cadmium on bioaccessibility of lead in spiked soils assessed by Unified BARGE Method. Chemosphere 154, 343-349.

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Cheng Peng	Edited the paper (5%)
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Mohammed Kader	Carried out experiments (20%)
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Jack C. Ng	Designed experiments (20%) Edited the paper (10%)

Contributions by others to the thesis

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Keywords

Mixed contamination, contaminated soil, bioaccessibility, soil property, HepG2 cells, interaction, risk assessment

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FoR code: 0502, Environmental Science and Management, 90%

FoR code: 1117, Public Health and Health Services, 10%

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List of Abbreviations

Al	aluminium
As	arsenic
ATCC	American Type Culture Collection
B[a]P	benzo[a]pyrene
BARGE	Bioaccessibility Research Group of Europe
BSA	bovine serum albumin
Cd	cadmium
CEC	cation exchange capacity
Cr	chromium
Cu	copper
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EC	electrical conductivity
EDTA	ethylenediaminetetraacetic acid
enHealth	Environmental Health Standing Committee
GC	gas chromatography
LC	liquid chromatography
Fe	iron
HCl	hydrogen chloride
Hg	mercury
HIL	health investigation level
HNO3	nitric acid
IARC	International Agency for Research on Cancer
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
IQ	intelligence quotient
LC50	lethal dose at which 50% of the population if killed in a given period of time
LOD	limit of detection
Mn	manganese
NAP	naphthalene
NEPM	National Environmental Protection Measure
Ni	nickel
NIST	National Institute of Standards and Technology
PAHs	polycyclic aromatic hydrocarbons
Pb	lead
PBS	phosphate-buffered saline
PHE	phenanthrene
PVDF	polyvinylidene fluoride
PYR	pyrene
QA	quality assurance
QC	quality control

SD	standard deviation
SRM	standard reference material
TC	total carbon
TN	total nitrogen
TS	total sulfur
U.S.	the United States
U.S. EPA	United States Environmental Protection Agency
UBM	Unified BARGE Method
Zn	zinc

Chapter 1 General introduction

Sites are usually contaminated with a mixture of metal/metalloids. Assuming additive effects is far from reflecting the real situation where possible interaction may take place among mixed contaminants. Oral ingestion is considered as a key route for soil contaminants, however, it remains unclear whether contaminants may interact with each other after being ingested thereby affecting their solubility in human digestive system or accumulation in the target organ, such as liver. This chapter describes the research background of the current study as well as providing an outline of this thesis.

1.1 Site contamination

Last century has witnessed the thrive of industry, such as gas works, manufacture, metal processing, tanneries, mining and landfill, which left behind a vast number of contaminated sites. Especially in Australia, 150,000 sites are subjected to potential contamination, according to senior researchers (Carbonell, 2013). Less than 1% of these contaminated sites have been remediated. Moreover, 60 to 80% of these sites are located in the urban environment, making this issue even more concerning. Heavy metal/metalloids are a group of hazards that have attracted lots of attention due to their toxicity, ubiquity and persistence (Wuana and Okieimen, 2011).

By reviewing the literature, it is found that soils on the sites are barely polluted with single metal/metalloid. For example, both Cd and Zn were released to the environment during metal (Zn) mining and smelting process since Cd sulfide co-occurs with the important Zn ore (Zn sulfide) (Grøn, 2003). On some occasions, organic compounds, such as PAHs were reported to exist as co-contaminants with metal/metalloids due to similar sources of contamination or materials and facilities used on the site (Hwang and Cutright, 2002; Wang et al., 2004; Thavamani et al., 2011). Without site-specific data, additive effects are usually assumed which may overestimate or underestimate the risk associated with mixed contaminants.

1.2 Metal/metalloid contaminants of concern

A variety of metal/metalloids were commonly detected at contaminated sites, including As, Cd, Cr, Cu, Hg, Mn, Ni, Pb and Zn, among which As, Cd and Pb are selected as prioritised metals/metalloid to be studied in this thesis not only because of the frequency that they co-

present in contaminated soils but also due to their well-documented health impacts on humans (ATSDR, 2007a, b, 2012). Metals/metalloid discussed in this thesis is referred to as As (metalloid), Cd (metal) and Pb (metal), specifically. The followings are a brief introduction of these three metals/metalloid in terms of source, fate in the environment, exposure pathways and adverse health effects.

1.2.1 Arsenic (As)

Arsenic is a naturally occurring element which distributes widely in the earth's crust and it is usually found in minerals and ores containing Cu, Fe, Pb and Zn. Categorised as a metalloid, As possesses both properties of metal and non-metal (Vernon, 2013). Natural processes such as volcanic activity and weathering of exposed mineralisation can release As into the environment. Emissions from anthropogenic activities, including mining and smelting, coal combustion, pesticide application, wood combustion and waste incineration, far exceeds those from natural sources (ATSDR, 2007a). Most As released to the environment ends up in soil or sediment. Humans can be exposed to As through food, water and air, of which food is usually the largest source. Children may also be exposed to As by ingesting soil or dust (Calabrese et al., 1989; Calabrese et al., 1997). The most reliable method of detecting recent short-term As exposure is measuring As in human urine. Hair or fingernails are better indicators for high-level exposure of As over the past 6-12 months.

Arsenic is a potent toxicant that may exist in several oxidation states, typically –1, +3, +5 (Carapella, 1992). Furthermore, As compounds are clarified as inorganic (compounds without an arsenic-carbon bond) and organic (compounds with an arsenic-carbon bond). It is generally accepted that inorganic As is more toxic than organic forms considering the arsenic-carbon bond is quite strong which limits the activity of As in solution. Existing information on health effects of inorganic and organic As is illustrated in Figures 1.1 (inorganic) and 1.2 (organic), which indicates extensive human health injuries are caused by inorganic As with little data being available for effects of organic As. IARC (International Agency for Research on Cancer) classifies As as Group 1 carcinogen regarding the evident relationship between exposure to As and human cancer. Also, U.S. EPA (The United States Environmental Protection Agency) has labelled inorganic As taken by inhalation and oral routes as Group A carcinogen.

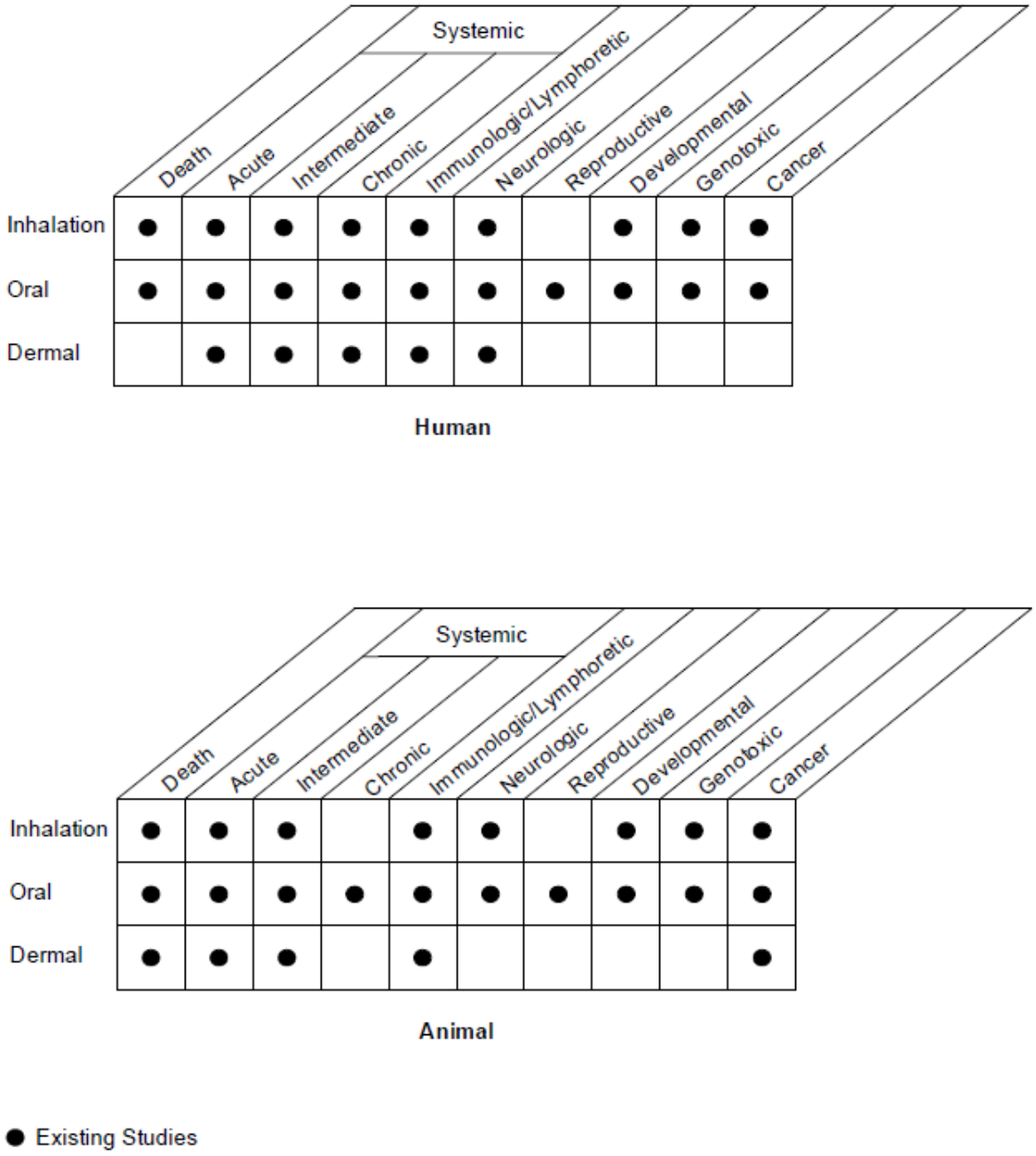

Figure 1.1 Available information on health effects of inorganic arsenic (ATSDR, 2007a)

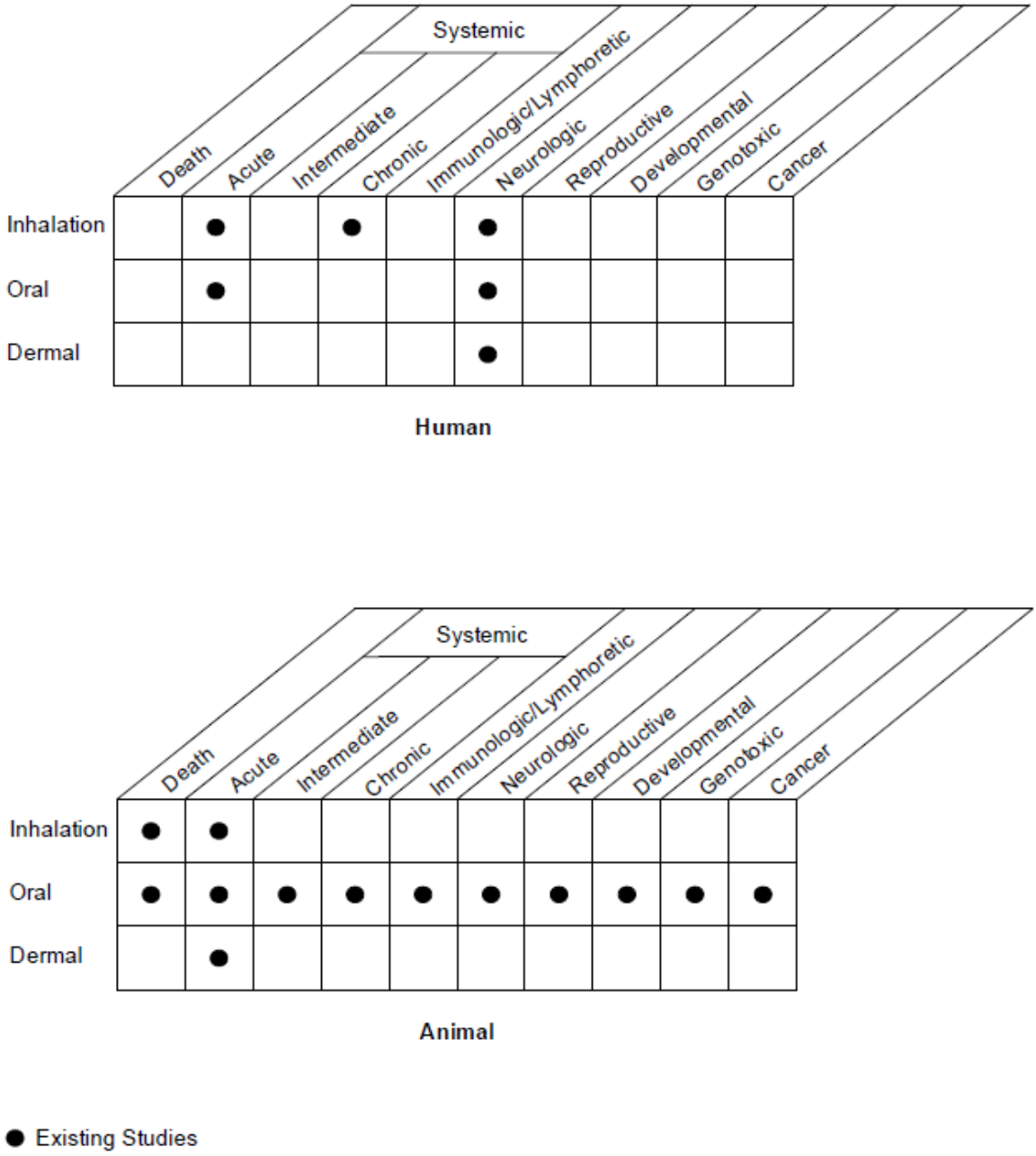

Figure 1.2 Available information on health effects of organic arsenic (ATSDR, 2007a)

1.2.2 Cadmium (Cd)

Widely distributed but sparsely found in the earth's crust at a concentration of 0.1-0.5 mg kg^{-1}, Cd often appears as carbonate or sulfide minerals combined with Zn ores, Zn-bearing Pb ores or complex Cu-Pb-Zn ores (Morrow, 2001). Natural activities such as volcanic eruptions, forest fires, generation of sea salt aerosols, result in Cd entering the environment via various biogeochemical cycles (Morrow, 2000; Shevchenko et al., 2003). Cadmium is

also introduced into air, water and soil through nonferrous metal mining and refining, manufacture and application of phosphate fertiliser, fossil fuel combustion, as a co-contaminant with Zn additives in lubricating oils and vehicle tyres, and waste incineration and disposal (ATSDR, 2012). Most (80-90%) of Cd is expected to partition to soils as its final fate. Potential exposure to Cd includes ingestion of food and drinking water, inhalation of particulates from ambient air or tobacco smoke, or ingestion of contaminated soil or dust, of which food (e.g. leafy vegetables, staples, peanuts, soybean, sunflower seed) is the most crucial one (ATSDR, 2012). After entering the human body, Cd is widely distributed, most of which is retained in the liver and kidney. One of the key characteristics of Cd toxicity is that it accumulates throughout the entire lifetime without significant excretion.

Cadmium toxicity has been first documented in the 1850s (Nordberg, 2009). The existing health effects of Cd are illustrated in Figure 1.3. The most well-known epidemiological study concerning Cd toxicity is the occurrence of Itai-Itai disease in Japan which resulted from the consumption of rice containing high levels of Cd (Wikipedia). Victims suffered from severe pains in the spine and joints. By integrating available information, IARC classified Cd as carcinogenic to humans (Group 1). U.S. EPA has endorsed Cd as a probable human carcinogen by inhalation (Group B1), based on its assessment of limited evidence of an increase in lung cancer in humans and sufficient evidence of lung cancer in rats.

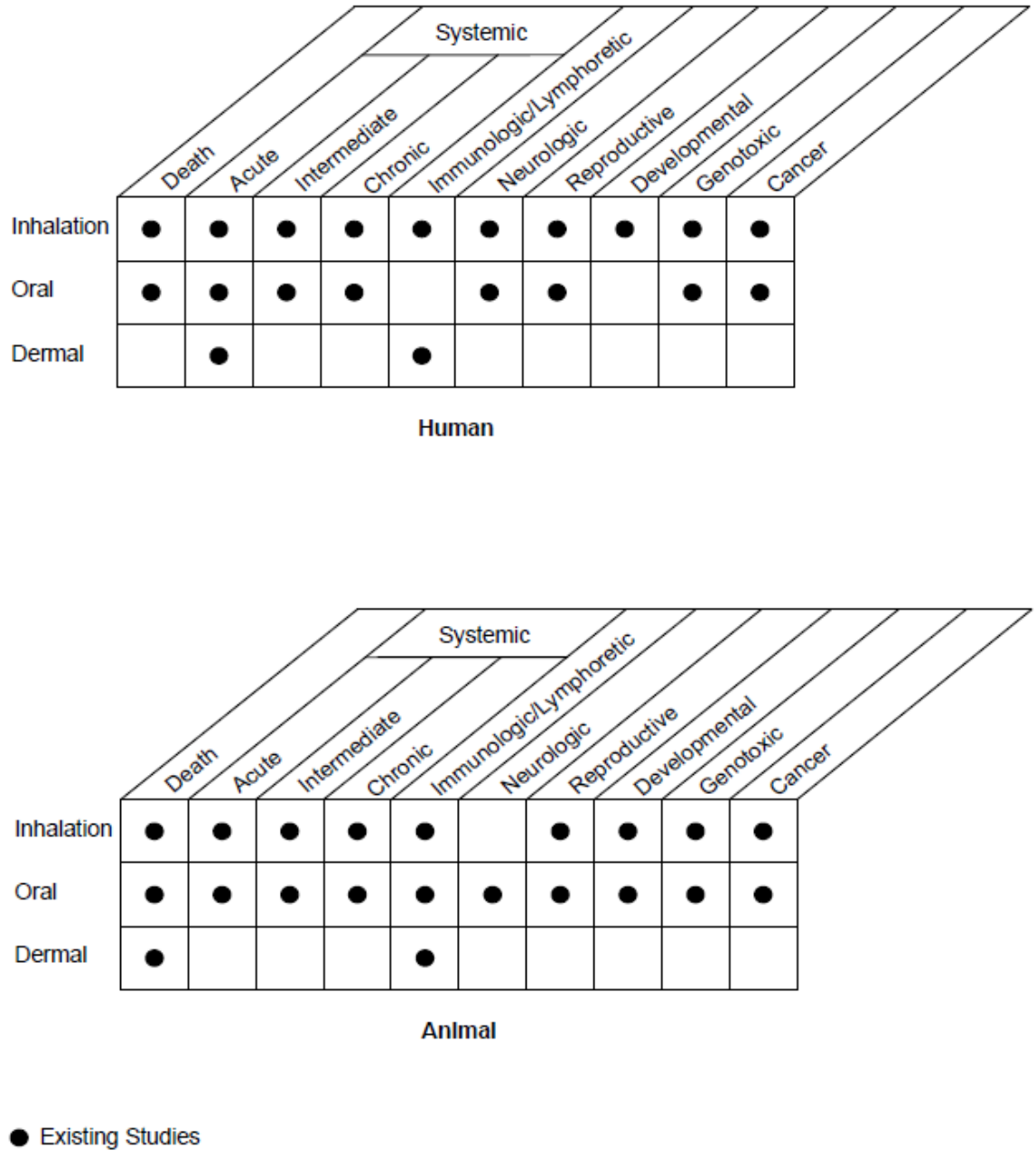

Figure 1.3 Available information on health effects of cadmium (ATSDR, 2012)

1.2.3 Lead (Pb)

Lead is found naturally in the earth's crust and usually combined with two or more other elements to form Pb compounds. In the environment, Pb is dominantly in the divalent state (Pb II) and is released to the environment primarily via anthropogenic activities, such as ore mining and smelting, combustion of coal and oil, manufacture of Pb-containing products, and waste incineration. For the past three centuries, Pb in the environment has increased three orders of magnitude as a result of human activities. The primary source of Pb in the

environment has historically been anthropogenic emissions to the atmosphere (Boggess and Wixson, 1977).

Lead dispersion in the air is commonly of particle form and is scavenged by rain or gravity settling. Soil and sediment seem to be primary sinks for Pb in the air. Lead which sinks in soil and sediment is so attached that it usually stays in the upper layers of soil and sediment, thus it is not easy to be flushed away or leaches into subsoil and groundwater (ATSDR, 2007b). Different Pb compounds can be transformed into other forms in the environment, but they cannot be destroyed or degraded, for which reason Pb derived from the legacy of previous uses is able to accumulate and persist in the environment over geological time.

Lead finds its way into humans mainly by exposure via ingestion and inhalation with the latter being much less compared to ingestion. During an early stage, Pb combines with proteins in the blood and is carried through the body, leading to its distribution in many tissues and organ systems. Over 95% of total Pb body burden is stored in bone for adults whereas about 70% is stored for children (Barry and Mossman, 1970).

Toxicological studies and epidemiological investigations have reached the conclusions that Pb has the potential to cause death, systemic effects, neurological effects, immunological and lymphoreticular effects, reproductive effects and developmental effects (Figure 1.4). In previous studies (Cooper et al., 1985; Cooper, 1988), increased mortality rate has been observed among male workers occupationally exposed to Pb at battery plants. Another issue related to neurological effect of Pb in adults was reported in 1987 (Kumar et al., 1987). Even more concerning, an inverse relationship between blood Pb concentration and IQ score has been detected in children (Lanphear et al., 2005).

Compared to As and Cd, evidence is inadequate to conclude that Pb is potentially carcinogenic to humans, but it still raises public concern considering Pb exposure in human life is everywhere, from household stuff such as kid toys and jewellery, old Pb-based paint, Pb-glazed ceramics and cosmetics to contaminated air, soil and water (ATSDR, 2007b). Therefore, the accumulative hazardous effect of Pb should not be ignored.

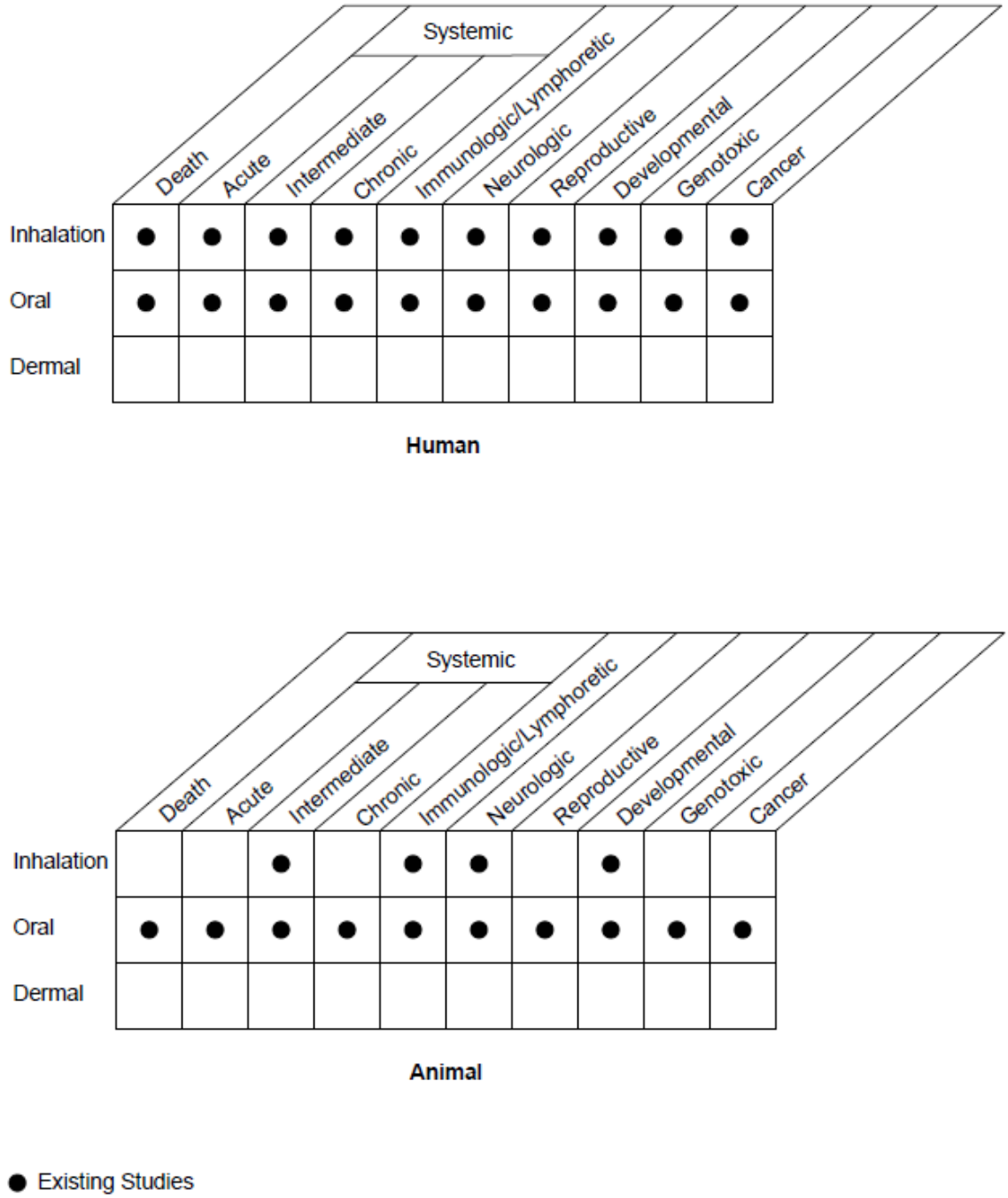

Figure 1.4 Available information on health effects of lead (ATSDR, 2007b)

1.3 Exposure assessment for contaminated sites

When coping with contaminated sites, risk assessment should be undertaken for the purpose of evaluating the degree of contamination, remediation or redevelopment. Risk assessment is defined as "the process of estimating the potential impact of a chemical, physical,

microbiological or psychosocial hazard on a specified human population or ecological system under a specific set of conditions and for a certain timeframe" (enHealth, 2012b). In 2002, the Australian enHealth Council has published a document (with updates in 2012), namely, *Guidelines for Assessing Human Health Risks From Environmental Hazards*, with the aim to provide national instruction for risk assessment (enHealth, 2012b). Specifically for the assessment of site contamination, a national guidance document, *the National Environment Protection (Assessment of Site Contamination) Measure 1999*, was released and later amended in 2013 (NEPC, 2013c). In Schedule B4 (*Guideline on Site-specific Health Risk Assessment Methodology*) of this document, the framework of risk assessment proposed by enHealth was adapted in order to provide specific guidance to contaminated lands (Figure 1.5). In this framework, exposure assessment is an important component which mainly involves identification of potential exposure pathway, estimation of exposure concentration of each pathway and estimation of contaminant intake for each pathway.

Soil contaminants can find their way to human body via ingestion, inhalation and dermal contact. ATSDR (2012) summarized the potential exposure scenarios as described below.

(1) Inhalation: inhalation of air-borne particles and cigarette smoking.

(2) Ingestion: incidental soil ingestion; dietary exposure including but not limiting to water, fruit, vegetables, grains, meat, milk, eggs and human breast milk.

(3) Dermal contact: dermal contact with soil, aerosol or water.

Among these exposure pathways, ingestion of soil is considered as an important exposure pathway in this thesis for the following reasons. In Australia, it is generally assumed the most sensitive group is 2-3 years old children (enHealth, 2012a). Due to their physiological behaviours and lower height, children aged at 2-3 years old are more likely to ingest soils than adults. Especially for children with soil-pica behaviour and geophagy, default values of 1g/day for children with soil-pica behaviour and 50g/day for geophagic children are assumed (Kahn and Stralka, 2009). Even for non-pica, a young child would incidentally ingest about 100-200 mg of soil per day which is 5-10 time higher than an adult (EPA, 2011; enHealth, 2012a). Furthermore, as mentioned previously, 60 to 80% of Australian contaminated sites are distributed in the urban area, which increases the possibility of soil ingestion via outdoor activities. Therefore, oral ingestion is selected as a key pathway to be investigated in this study.

Estimating the oral intake of soil contaminants is based on the exposure concentrations and frequencies. Exposure concentrations are derived from the direct measurement of total concentrations in soils wherever possible as suggested in Australian guidelines (NEPC, 2013b). However the calculation utilising total concentration suffers from the limitation that assuming the amount of intake is 100% absorbed to human bloodstream. This limitation becomes more obvious when soil components (e.g. organic matter, minerals) strongly bind with metal/metalloids thus reducing their solubility in human gastrointestinal tract (Park et al., 2011). Therefore, this methodology of estimation could overestimate health risk and increase the associated remediation budget.

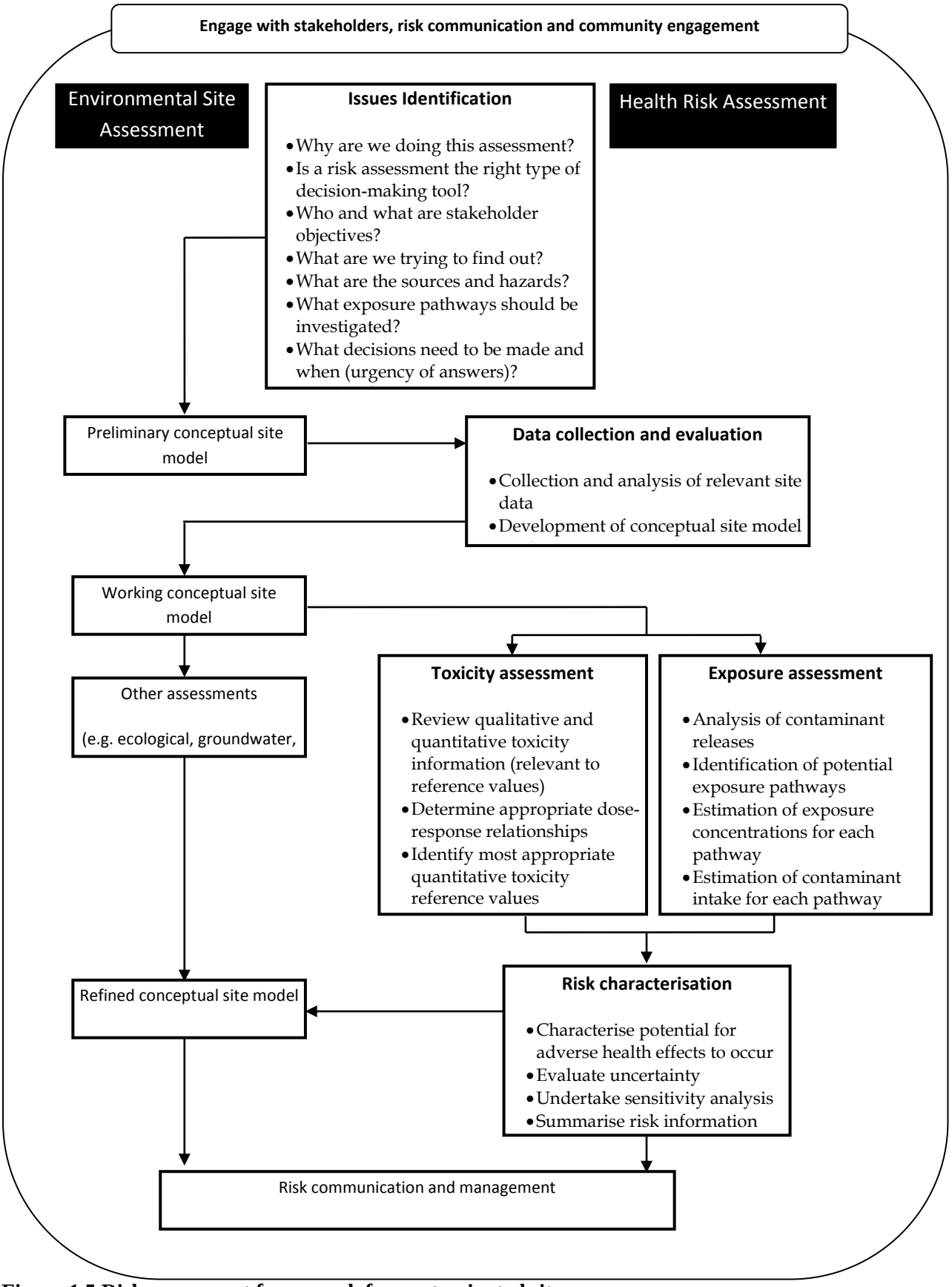

Figure 1.5 Risk assessment framework for contaminated sites

1.4 Incorporating bioavailability and bioaccessibility into exposure assessment

1.4.1 Bioavailability

As discussed in Section 1.3, there may be a difference between the amount of contaminant being ingested and the amount being absorbed, which leads to increasing relevance to the term, bioavailability. The definition of bioavailability varies according to different disciplines. In the context of human risk assessment and exposure pathway of ingestion, bioavailability (absolute bioavailability) is referred to as the fraction of an orally administered dose of the chemical that reaches the systemic circulation (Ng et al., 2013). Relative bioavailability is defined as the ratio of the bioavailability of a substance in one exposure context (e.g. soil) to that in another exposure context (usually the chemical administered in a convenient dosing vehicle in an experimental study). Historically, bioavailability has been measured by the methods of experimental animal models, validated toxicokinetic models or human studies (Grøn, 2003). Available animal models include cattle, dog, rat, swine, rabbit, monkey as reviewed (Ng et al., 2013). Among these animal models, juvenile swine is regarded as a good representative of children's physiological conditions (Weis and Lavelle, 1991).

1.4.2 Bioaccessibility

Performing *in vivo* bioavailability experiments on a site-specific basis is not practical due to the cost, labour and especially, animal ethical issues. Therefore, *in vitro* bioaccessibility testing systems have been established to predict bioavailability. Bioaccessibility represents the amount of contaminant released from soil and available for absorption in human gastrointestinal tract (Ng et al., 2015), which is the key process limiting the bioavailability. These bioaccessibility-measuring systems do not aim to replicate the exact conditions in human digestive system but mimic key processes. Bioaccessibility methods that cover one or more of our target elements have been previously reviewed (Basta and Juhasz, 2014b; Ng et al., 2015).

Generally, up to three phases (oral cavity, stomach and intestine) are incorporated into these methods. Oral cavity is optional due to short residence time whilst gastric and intestinal phases are commonly included. Table 1.1 shows a list of *in vitro* methods which are categorised according to the incorporation of different phases.

Table 1.1 *In vitro* bioaccessibility methods

Included phases	Abbreviate	Full name	Reference
Gastric phase only	RBALP	Relative Bioaccessibility Leaching Procedure	(Brattin et al., 2013)
	SBET	Simplified Bioaccessibility Extraction Test	(Juhasz et al., 2007b)
	USEPA 9200	Standard Operating Procedure for an In Vitro Bioaccessibility	(EPA, 2012)
Gastric + intestinal phases	PBET	Physiologically Based Extraction Test	(Ruby et al., 1996)
	SBRC	Solubility Bioaccessibility Research Consortium	(Kelley et al., 2002)
	IVG	In Vitro Gastrointestinal Extraction Test	(Rodriguez and Basta, 1999)
	OSU IVG	Ohio State University In Vitro Gastrointestinal Extraction Test	(Basta et al., 2007)
	DIN	Standarlised German In Vitro Assay	(DIN, 2000)
Saliva +gastric +intestinal phases	RIVM	Dutch National Institute for Public Health and the Environment	(Oomen et al., 2003a)
	UBM	Unified Bioaccessibility Research Group of Europe Method	(Denys et al., 2012)
Gastric and intestinal phases(small and large intestines, colon descendens, intestinal microflora)	SHIME	Simulator of the Human Intestinal Ecosystem	(Oomen et al., 2002)
	TIM	Dynamic Computer-controlled Gastrointestinal Model	(Minekus M, 1995)

For each method, parameters may vary in the aspects of chime composition (e.g. pepsin, glycine, mucin, bile), gastric and intestinal phase pH, existence of food, residence time, soil/solution ratio, redox, temperature, agitation, etc. In Table 1.2, a number of commonly investigated bioaccessibility methods are listed in details. Among these methods, the Unified BARGE Method (UBM) is highly physiological-related by comprising three key cavities (mouth, stomach and intestinal) and key ingredients of human digestive fluids (e.g. mucin, pepsin, amylase, bile).

Table 1.2 Composition and parameters in commonly utilised *in vitro* bioaccessibility assays.

In vitro assay	Phase	Composition (g L^{-1})	Soil/solution ratio	pH	Extraction time (h)
RBALP	Gastric	30.03 g glycine	1:100	1.5	1
SBRC	Gastric	30.03 g glycine	1:100	1.5	1
	Intestinal	1.75 g bile, 0.5 g pancreatin	1:100	7	4
IVG	Gastric	10 g pepsin, 8.77 g NaCl	1:150	1.8	1
	Intestinal	3.5 g bile, 0.35 g pancreatin	1:150	5.5	1
PBET	Gastric	1.25 g pepsin, 0.5 g sodium malate, 0.5 g sodium citrate, 420 µL lactic acid, 500 µL acetic acid	1:100	2.5	1
	Intestinal	1.75 g bile, 0.5 g pancreatin	1:100	7	4
DIN	Gastric	1 g pepsin, 3 g mucin, 2.9 g NaCl, 0.7 g KCl, 0.27 g KH_2PO_4	1:50	2	2
	Intestinal	9.0 g bile, 9.0 g pancreatin, 0.3 g trypsin, 0.3 g urea, 0.3 g KCl, 0.5 g $CaCl_2$, 0.2 g $MgCl_2$	1:100	7.5	6
UBM	Saliva	0.896 g KCl, 0.888 g NaH_2PO_4, 0.2 g KSCN, 0.57 g Na_2SO_4, 0.298 g NaCl, 1.8 mL of 1 M NaOH, 0.2 g urea, 0.145 g amylase, 0.05 g mucin, 0.015 g uric acid	1:15	6.5	few mins
	Gastric	Gastric phase (pH 0.9–1.0): 2.752 g NaCl, 0.266 g NaH_2PO_4, 0.824 g KCl, 0.4 g $CaCl_2$, 0.306 g NH_4Cl, 8.3 mL of 37 % HCl, 0.65 g glucose, 0.02 mg glucuronic acid, 0.085 g urea, 0.33 g glucosaminehydrochloride, 1 g bovine serum albumin, 3 g mucin, 1 g pepsin	1:37.5	1.2	1
	Intestinal	Duodenal phase (pH 7.4±0.2): 7.012 g NaCl, 5.607 g $NaHCO_3$, 0.08 g KH_2PO_4, 0.564 mg KCl, 0.05 g $MgCl_2$, 0.18 ml of 37 % HCl, 0.1 g urea, 0.2 g $CaCl_2$, 1 g bovine serum albumin, 3 g pancreatin, 0.5 g lipase; Bile phase (pH 8.0±0.2): 5.259 g NaCl, 5.785 g $NaHCO_3$, 0.376 g KCl, 0.18 mL of 37 % HCl, 0.25 g urea, 0.222 $CaCl_2$, 1.8 g bovine serum albumin, 6 g bile	1:100	6.5	4

The acceptance of these methods in human risk assessment highly depends on whether good correlation exists between bioaccessibility data and bioavailability data (Basta and Juhasz, 2014b). As shown in Table 1.3, researches have aimed to explore an *in vitro* bioaccessibility system which can predict *in vivo* data. As can be seen in the table, different bioaccessibility systems have been investigated to correlate with different *in vivo* models for different metal/metalloids. Among these methods, the UBM has been statistically related with

bioavailability data of a juvenile swine model (a good representative of the gastrointestinal physiology of children) for the targeted metals/metalloid in this thesis, i.e. As, Cd and Pb (Denys et al., 2012). Moreover, endeavours have been made to assess and improve the repeatability and reproducibility of the UBM by undertaking inter-laboratory trials (Wragg et al., 2011). Therefore, UBM is selected for bioaccessibility measurement in the current study.

Table 1.3 *In vivo* bioavailability and *in vitro* bioaccessibility correlation studies.

In vitro assay	Metal/ metalloid	Animal model	Soil sources	Reference
IVG	As	Swine	Mining sites	(Rodriguez and Basta, 1999)
	As	Swine	Railway corridors, dip sites, mine sites, gossans	(Juhasz et al., 2009b)
	As	Swine	Mining- and herbicide-impacted sites	(Juhasz et al., 2015)
	As	Swine	Smelter-contaminated sites	(Basta et al., 2007)
	As	Mouse	Urban residential, residential and smelter slag sites	(Juhasz et al., 2014b)
	As	Mouse	Farming, mining, and smelting sites	(Li et al., 2015b)
	As	Mouse	Household dust	(Li et al., 2014b)
	Cd	Swine	Hazardous waste sites	(Schroder et al., 2003)
	Cd	Mouse	Defense, industrial, and mine sites	(Juhasz et al., 2010)
	Cd	Mouse	Mining, farming, smelting, industry, residential sites	(Li et al., 2016b)
	Pb	Swine	Hazardous waste sites	(Schroder et al., 2004)
	Pb	Mouse	Household dust	(Li et al., 2014a)
	Pb	Mouse	Farming, mining, smelting sites	(Li et al., 2015a)
	Pb	Minipig	Urban areas and around former mine or smelting industries	(Marschner et al., 2006)
PBET	As	Swine	Railway corridors, dip sites, mine sites, gossans	(Juhasz et al., 2009b)
	As	Swine	Mining- and herbicide-impacted sites	(Juhasz et al., 2015)
	As	Mouse	Farming, mining, smelting sites	(Li et al., 2015b)
	As	Rabbits/ Monkey	Residential soils, house dust	(Ruby et al., 1996)
	As	Mouse	Urban residential, residential and smelter slag sites	(Juhasz et al., 2014b)
	As	Mouse	Household dust	(Li et al., 2014b)
	Cd	Mouse	Mining, farming, smelting, industry, residential sites	(Li et al., 2016b)
	Cd	Mouse	Defense, industrial, and mine sites	(Juhasz et al., 2010)
	Pb	Sprague-Dawley rats	Mining waste materials, residential soils, tailings, stream channel samples	(Ruby et al., 1996)
	Pb	Mouse	Household dust	(Li et al., 2014a)

Table 1.3 (continued)

	Pb	Mouse	Farming, mining, smelting sites	(Li et al., 2015a)
RBALP	As	Swine/monkey	Mining, smelting, and pesticide or herbicide-impacted sites	(Brattin et al., 2013)
	Pb	Swine	Small-arms ranges	(Bannon et al., 2009)
	Pb	Swine	Residential soils, tailings, and slags from mining-related waste sites	(Drexler and Brattin, 2007)
	Pb	Quail	U.S. EPA Superfund sites	(Beyer et al., 2016)
	Pb	Mouse	Shooting range, incinerator waste, former landfill, smelting, mining sites	(Smith et al., 2011a)
SBRC-G	As	Swine	Mining and herbicide-impacted sites	(Juhasz et al., 2014a)
	As	Swine	Railway corridors, dip sites, mine sites, gossans	(Juhasz et al., 2007a)
SBRC	As	Swine	Railway corridors, dip sites, mine sites, gossans	(Juhasz et al., 2009b)
	As	Swine	Mining- and herbicide-impacted sites	(Juhasz et al., 2015)
	As	Mouse	Mining, orchard, railway corridor, cattle dip, gossan	(Bradham et al., 2015)
	As	Mouse	Household dust	(Li et al., 2014b)
	As	Mouse	Urban residential, smelter slag sites	(Juhasz et al., 2014b)
	As	Mouse	Farming, mining, smelting sites	(Li et al., 2015b)
	As	Mouse	Urban residential, smelter slag, residential sites	(Bradham et al., 2011)
	Cd	Mouse	Defense, industrial, and mine sites	(Juhasz et al., 2010)
	Cd	Mouse	Mining, farming, smelting, industry, residential sites	(Li et al., 2016b)
	Pb	Swine	Urban residential, domestic incinerator	(Juhasz et al., 2009)
	Pb	Mouse	Household dust	(Li et al., 2014a)
	Pb	Mouse	Farming, mining, smelting sites	(Li et al., 2015a)
	Pb	Mouse	Shooting range, incinerator waste, former landfill, smelting, mining sites	(Smith et al., 2011a)
	Pb	Mouse	Parks	(Li et al., 2016a)
DIN	As	Swine	Railway corridors, dip sites, mine sites, gossans	(Juhasz et al., 2009b)
	As	Swine	Mining- and herbicide-impacted sites	(Juhasz et al., 2015)
	As	Mouse	Household dust	(Li et al., 2014b)
	As	Mouse	Urban residential, smelter slag sites	(Juhasz et al., 2014b)
	As	Mouse	Farming, mining, smelting sites	(Li et al., 2015b)
	Cd	Mouse	Defense, industrial, and mine sites	(Juhasz et al., 2010)
	Pb	Mouse	Household dust	(Li et al., 2014a)

Table 1.3 (continued)

UBM	As	Swine	Mining, smelting sites	(Denys et al., 2012)
	As	Swine	Railway corridors, dip sites, mine sites, gossans	(Juhasz et al., 2011a)
	As	Swine	Mining- and herbicide-impacted sites	(Juhasz et al., 2015)
	As	Mouse	Farming, mining, smelting sites	(Li et al., 2015b)
	As	Mouse	Urban residential, smelter slag sites	(Juhasz et al., 2014b)
	Cd	Swine	Mining, smelting sites	(Denys et al., 2012)
	Cd	Mouse	Mining, farming, smelting, industry, residential sites	(Li et al., 2016b)
	Pb	Swine	Mining, smelting sites	(Denys et al., 2012)
	Pb	Mouse	Farming, mining, smelting sites	(Li et al., 2015a)

A wide range of factors can influence the bioaccessibility results, including soil properties, mineralogy, soil-metal/metalloid contacting time, *in vitro* assays, source of contamination, natural weathering/aging (Juhasz et al., 2007b; Sarkar et al., 2007; Meunier et al., 2010; Roussel et al., 2010; Das et al., 2013; Walraven et al., 2015). For laboratory spiked soils extracted by specific *in vitro* methods (e.g. UBM in this thesis), soil properties and mineralogy play more prudent roles than other factors. Throughout the literature, a number of soil physicochemical are commonly reported to have positive or negative effects on bioaccessibility of As, Cd and Pb, such as metal oxides, pH, organic matter, clay contents, phosphate, etc. Metal oxides (usually Fe/Al/Mn oxides) in soils provide sorption sites for binding metal/metalloids. For example, it is well known that Fe oxide is primary sorbent for As in soils (Yang et al., 2002; Juhasz et al., 2007b; Sarkar et al., 2007). Arsenic becomes less bioaccessible by either establishing inner-sphere complex or precipitating with Fe oxide in soils. Due to the high affinity of As to Fe oxide, Fe oxide materials have been applied in the remediation of As-contaminated soils (Komarek et al., 2013). pH exhibits distinct effects on Cd/Pb and As. In low pH environment, sorption of positive-charged Cd and Pb in soils would decrease due to the increased amount of H^+ competing for binding sites thereby bioaccessibility of Cd and Pb increases (Hettiarachchi and Pierzynski, 2004). On the contrary, low pH is reported to enhance the absorption of negatively-charged As on Fe oxide thus reducing bioaccessible As since Fe oxide would be become more positively-charged at low pH (Juhasz et al., 2007b). Soil organic matter is known to possess ligands or functional groups which can chelate metal/metalloids, therefore decreasing bioaccessibility (Harter and Naidu, 1995). For example, organic matter was observed as the main sink for Pb in organic-

rich soils (Morin et al., 1999). Clay-size soil particles are characterized as large surface area and internal porosity which can easily trap metal/metalloids in the pore network, thereby exerting negative effect on bioaccessibility (Sarkar et al., 2007). Soil mineral content has been widely reported as a determining factor for Pb bioaccessibility by affecting Pb speciation in soils (Bosso and Enzweiler, 2008; Lu et al., 2011; Smith et al., 2011b). Mineral forms of Pb sulfide and Pb phosphate are much less bioaccessible than Pb carbonate and Pb oxides (Ruby et al., 1996; Zia et al., 2011). Therefore, phosphate materials have long been utilised as Pb immobilizer in soil (Miretzky and Fernandez-Cirelli, 2008).

Soil-bioaccessibility relationships are usually site-dependent and cannot be applied universally. For instance, total organic carbon was observed to have a negative effect on As bioaccessibility in a study conducted in India (Das et al., 2013) but positive correlation was obtained between As bioaccessibility and total organic carbon in a study based on Canadian soils (Girouard and Zagury, 2009). Limited data are available to demonstrate effects of soil characteristics on the bioaccessibility of As, Cd and Pb in Australian soils. Since Australia is a geographically-isolated continent which may possess unique natural environment, relationships between bioaccessibility and soil properties should be further investigated. The focus of this study is on the effects of commonly-reported soil properties (e.g. metal oxide, organic matter, pH, clay) on bioaccessibility of As, Cd and Pb.

1.4.3 Bioaccessibility of mixed contaminants

As mentioned previously, it is seldom that one metal/metalloid contaminant exists at contaminated sites. Literature has indicated the possible interactions among As, Cd and Pb in soils with most studies focusing on binary mixtures. For instance, Pb was reported to be retained in soils stronger than Cd which may be attributed to its greater hydrolysis constant, higher atomic weight and ionic radius, and larger Misono softness value over Cd (Shaheen, 2009). These parameters favoured Pb to more readily undergo inner-sphere surface sorption and complexation than Cd. Therefore, competition for sorption sites have been observed between Cd and Pb (Serrano et al., 2005; Appel et al., 2008). Lead and As commonly co-exist at contaminated sites as a result of lead arsenate (PbHAsO4) being used as pesticide in orchids and their co-occurrence in mineralised ore materials (Arai et al., 2006; Vaca-Escobar et al., 2012). Lead arsenate is highly likely to be dissociated into ionic species in the acidic gastric phase as it is soluble in acid, but exists as an insoluble compound in the alkaline

conditions of the intestinal phase. A number of studies have reported that the presence of one metal/metalloid may affect the bioaccessibility of another metal/metalloid. For instance, oral bioaccessible Pb in soil was reported to increase along with the increase in total Cd concentration in soil (Pelfrene et al., 2011). Also, water extractable As was found to be crucial for controlling bioaccessible Pb in the gastric and small intestinal phase (Cui and Chen, 2011). These previous researches point out the possibility of interaction in terms of bioaccessibility. However, there are no detailed studies which elucidate interaction effects of As, Cd and Pb (target contaminants in this thesis) on their respective bioaccessibility in different types of soils.

There are two scenarios where multiple contaminants are aged in the environment: (1) independent ageing: contaminants are aged in different spots or there is a long time interval between different contaminants entering the same spot; (2) simultaneous ageing: contaminants enter the same soil and are aged concurrently. Mixed contaminants can co-exist in human gastrointestinal tract when children ingest independently-aged contaminants (e.g. children take in As-containing soil at one spot followed by ingestion of Cd-bearing soil) or simultaneously-aged contaminants. For independently-aged contaminants, the interaction can only take place in the simulated digestive system (e.g. forming insoluble compounds or competing for binding ligands) since they are not physically co-present in soils. For simultaneously-aged contaminants, the interaction may be observed either in soils (e.g. competing for sorption sites) or in the digestive system (e.g. forming insoluble compounds or competing for binding ligands). Therefore, these two exposure scenarios should both be investigated to fully understand the interaction effects of As, Cd and Pb on their respective bioaccessibility under different exposure conditions. Considering PAHs have been found to co-occur with As, Cd and Pb at some sites as discussed in Section 1.1, effects of PAHs on the bioaccessibility of As, Cd and Pb will be addressed in this study as well. Based on the frequency of co-occurrence and toxicity, naphthalene (NAP), phenanthrene (PHE), pyrene (PYR) and benzo[a]pyrene (B[a]P) were selected as representatives of PAHs to explore their effects on the bioaccessibility of As, Cd and Pb. These PAHs are 4 out of 16 PAH priority pollutants listed by U.S. EPA and are regarded as model PAHs (Gan et al., 2009). Emphasis would be laid on the interaction among mixtures of As, Cd and Pb.

1.5 *In-vitro* model to measure uptake interaction among As, Cd and Pb on hepatic level

After As, Cd and Pb in soils are solubilised in human digestive system, the distribution and accumulation of contaminants across the body is of great importance to identify related toxicity. Liver is well-known for its metabolic and detoxification functions and has been reported as the important organ for distribution and metabolism of As, Cd and Pb (Underhill, 1914; Chen et al., 2005; Arroyo et al., 2012). It is assumed that contaminants in the liver can reflect their overall systemic levels (Grøn, 2003) therefore liver is the key organ that should be considered when investigating the accumulation of As, Cd and Pb in human body. Previous *in vivo* experiments using rat suggest additive or antagonistic effects between binary mixtures of As, Cd and Pb (Fairhall and Miller, 1941; Mahaffey et al., 1981; Elsenhans et al., 1987; Yanez et al., 1991). However, due to time and cost constraint, ethical concerns, species difference, increasing numbers of chemicals and mixtures of chemicals, *in vitro* liver models have been established, including liver slices, immortalised cell lines, primary hepatocytes, three-dimension cell culture system, bioartificial livers, and co-culture systems (Soldatow et al., 2013). Immortalised cell lines and primary hepatocytes are the most widely-adopted *in vitro* models. HepG2 cell line is one of the well-characterised immortalised cells, which can activate and detoxify xenobiotics and thus reflect the metabolism of xenobiotics in the human body better than other metabolically incompetent cells utilised in conventional *in vitro* assays (Dehn et al., 2004; Mersch-Sundermann et al., 2004; Baderna et al., 2011; Baderna et al., 2013). To date, no study has explored the potential of HepG2 cells to mimic *in vivo* interaction among As, Cd and Pb during hepatic accumulation, i.e. whether the interaction pattern detected in *in vitro* HepG2 cells is consistent with that observed in animal models.

Transport pathways for As, Cd and Pb have been investigated across various cell types. For the hepatic transport of Cd, several mechanisms have been proposed. Previous studies indicate Ca^{2+} channels were involved in the hepatic uptake of Cd because of the similarity of Cd radius (0.95 Å) to that of Ca (1.00 Å) (Jacobson and Turner, 1980). Also, it is thought that Cd could be bound to protein and be taken into hepatocytes via receptor-mediated endocytosis. For example, For example, experimental evidence has shown that Cd can bind to ferritin and transferrin by replacing the position of Fe and then be transported into hepatocytes with the aid of DMT (divalent metal transporter) 1. Albumin, as the most abundant protein in plasma, may act as a carrier for Cd by forming Cd-albumin complex (DelRaso et al., 2003) . Regarding Pb, it is found that Pb can be transported into cells by means of Ca^{2+} channels, DMT 1, endocytosis of Pb-protein complexes, Ca^{2+} pumps and

anion exchanger (Bridges and Zalups, 2005), some of which are also utilised by Cd as mentioned before. Therefore, there might be possible that Cd and Pb would compete with each other for transporters when concentrations of Cd and Pb are over-saturated. In fact, *in vivo* data summarised by ATSDR (2004) mainly demonstrate antagonistic or additive effects of Cd and Pb on each other's hepatic accumulation. This interactive effect remains to be investigated in *in vitro* hepatocytes. Compared with Cd and Pb, arsenate possesses a different transport pathway. It is well known that As (V), having a similar atomic structure with phosphate, is taken up by phosphate transporters in mammals (Rosen and Liu, 2009; Zangi and Filella, 2012). Thereby it is seldom possible that Cd (or Pb) and As would interact through competition. However, formation of insoluble compound (cadmium arsenate) was observed in a previous *in vivo* study (Diacomanolis et al., 2014), which raises the curiosity that whether insoluble compounds such as cadmium/lead arsenate can be formed in *in vitro* culture system thus reducing metal/metalloid uptake into hepatocytes.

Moreover, throughout the literature, HepG2 cells were treated by adding chemicals solubilised in pure solutions (e.g. water, DMSO) into cell culture medium, which did not always reflect the real exposure situation, especially for soil contaminants. For example, soil contaminants may undergo a series of reactions (e.g. dissolution, absorption) in human digestive system and end up with several species. Therefore, in this study, HepG2 cells were exposed to soluble As, Cd or Pb extracted in the digestive system in order to understand the difference in the uptake between As/Cd/Pb in pure solution and in digestive fluids. Effects of PAHs on the uptake of As, Cd and Pb were also studied by exposing cells with mixtures of As/Cd/Pb and PAHs.

1.6 Aims and objectives

Soils are frequently contaminated with mixtures, such as As, Cd and Pb (or mixed with PAHs). Previous studies mainly focus on the effects of soil properties or mineralogy on the bioaccessibility of As, Cd and Pb without considering the interactive effects of As, Cd and Pb on their respective bioaccessibility. Moreover, no *in vitro* model is available to investigate post accumulation and interaction of As, Cd and Pb on hepatic level after digestive system. Therefore, this project aims to add new knowledge to exposure assessment of mixed contaminants with respect to bioaccessibility and hepatic accumulation. The objectives set forth in the current study were:

(1) To probe the key soil parameters governing bioaccessibility of As, Cd and Pb in Australian soils.

(2) To study the post ingestion interaction among As, Cd and Pb when As/Cd/Pb-bearing soils co-exist in simulated digestive system.

(3) To explore the potential of HepG2 cells being a useful *in vitro* model to study uptake and interaction of As, Cd and Pb on the hepatic level.

(4) To shed light on the effects of PAHs on the bioaccessibility of As, Cd and Pb as well as on the subsequent accumulation in hepatocytes.

1.7 Thesis framework

Chapter 1 introduces the background of this study as well key concepts regarding exposure assessment, bioavailability, bioaccessibility, hepatic accumulation. Chapter 2 aims to elucidate the key soil parameters controlling the bioaccessibility of As and Cd as well as their interaction during UBM extraction (independently-aged soils) whilst Chapter 3 is a follow-up work that probes the bioaccessibility of Pb in Australian soils and effects of As and Cd on bioaccessibility of Pb (independently-aged soils). In Chapter 4, binary or ternary mixtures of As, Cd and Pb were aged simultaneously and interaction effects of As, Cd and Pb on their respective bioaccessibility over time were assessed by UBM. Chapter 5 investigates the uptake of UBM-extracted As, Cd and Pb in HepG2 cells and their possible interactions during uptake. Chapter 6 summarises effects of PAHs (NAP, PHE, PYR and B[a]P) on the bioaccessibility and uptake in HepG2 cells of As, Cd and Pb. In the end, Chapter 7 provides a general discussion and conclusion of the present study and suggests the potential direction of future work.

Chapter 2 Bioaccessibility of arsenic and cadmium assessed for *in vitro* bioaccessibility in spiked soils and their interaction during the Unified BARGE Method (UBM) extraction

Chapter 2 was published in *Chemosphere* (doi:10.1016/j.chemosphere.2015.12.091)

Abstract

Recent decades have seen a growing popularity of *in vitro* bioaccessibility being utilised as a screening tool in human health risk assessment. However, the existing bioaccessibility studies only focus on the single contaminant. Considering humans are likely to ingest multi-contaminants, these contaminants could interact within human gastrointestinal tract, which may lead to an increase or decrease in bioaccessibility. In this study, seven different types of soil were spiked with As or Cd and aged for one year. The effects of soil properties on the bioaccessibility were examined. Moreover, the interaction between As and Cd in the simulated human digestive system was studied by mixing As-spiked soil with Cd-spiked soil of the same type during bioaccessibility test. Results show that the bioaccessibility of As ranged from 40±2.8 to 95±1.3% in the gastric phase and 16±2.0 to 96±0.8% in the intestinal phase whilst a significant difference was observed between Cd gastric bioaccessibility (72±4.3 to 99±0.8%) and intestinal bioaccessibility (6.2±0.3 to 45±2.7%). Organic carbon, Fe oxide and Al oxide were key parameters influencing the bioaccessibility of As (gastric and intestinal phases) and Cd (intestinal phase). No interactions between As and Cd during bioaccessibility test were observed in any type of soils, which indicates As and Cd may age independently and did not interact while being extracted during bioaccessibility test. Thus additive effect may be proposed when estimating the bioaccessibility of mixtures of independently-aged As and Cd in soils.

2.1 Introduction

Soil is considered as a sink for contaminants generated from various sources, including industrial activities, vehicle emissions, waste disposal and the weathering of building structures. Among inorganic contaminants, As and Cd have received significant public concern due to their toxicity, ubiquity and persistence. Humans can be exposed to these hazardous elements in soils via various routes among which ingestion is regarded as a significant pathway, especially for children aged from 2 to 6 years old due to their higher incidental ingestion rate via hand-to-mouth activities compared to that by adults (Calabrese et al., 1989; Rodriguez and Basta, 1999). Generally, human health risk assessment is based on total concentrations of soil contaminants, which assumes 100% of contaminants in soil, in the absence of site-specific data, can be absorbed into the systemic circulation through the gastrointestinal tract (100% bioavailability). However, metal/metalloids may have lower bioavailability if they combine with minerals, organic matter and other components in soil (Park et al., 2011). Thus only a proportion of metal/metalloids can be solubilised in human digestive system and available for further uptake, which is referred as bioaccessibility (Ruby et al., 1999; Ng et al., 2015). Assumption of 100% bioavailability may overestimate risks and increase clean-up costs at contaminated sites.

To overcome the conservatism of assuming 100% bioavailability, *in vivo* models (e.g. cattle, dog, rat, swine, rabbit, monkey) have been adopted to generate oral bioavailability data as described in a recent review (Ng et al., 2015). However, it is impractical to produce *in vivo* data for each specific site, especially with the global tendency to replace, reduce and refine animal experiments (the so-called 3R principles in animal ethics). Considering bioaccessibility is the key parameter limiting the bioavailability, *in vitro* bioaccessibility systems have been used to predict *in vivo* uptake after being correlated with *in vivo* animal bioavailability data (Ruby et al., 1996; Rodriguez and Basta, 1999; Juhasz et al., 2009; Denys et al., 2012).

Bioaccessibility can be influenced by a number of soil properties (Juhasz et al., 2007b; Sarkar et al., 2007; Meunier et al., 2010; Roussel et al., 2010; Das et al., 2013), e.g. metal oxides, pH, phosphate, organic carbon, particle distribution. Some of these soil-bioaccessibility relationships are consistent in the literature. For instance, it is well known that Fe oxide can immobilise As and reduce its bioaccessibility of As (Yang et al., 2002; Juhasz et al., 2007b; Sarkar et al., 2007). Due to the high affinity of As to Fe oxide, Fe oxide materials have been

applied in the remediation of As-contaminated soils (Komarek et al., 2013). But these soil property-bioaccessibility relationships are site-dependent in most cases. For instance, total organic carbon was observed to have a negative effect on As bioaccessibility in a study conducted in India (Das et al., 2013) but positive correlation was obtained between As bioaccessibility and total organic carbon in a study based on Canadian soils (Girouard and Zagury, 2009), which suggests other site-specific factors may also play a role. There are little published data which demonstrate effects of soil properties on the bioaccessibility of As and Cd in Australian soil. Since Australia is a geographically-isolated continent which may possess unique natural environment, relationships between bioaccessibility and soil properties should be further investigated.

Moreover, previous studies have only focused on the bioaccessibility of single metal/metalloid without considering the post-ingestion interaction between multiple metal/metalloids in human digestive system may influence their bioaccessibility. Our recently-published study shows the bioavailability of As (administered in aqueous solution) in rat was significantly reduced with the co-administration of Cd (Diacomanolis et al., 2014). The formation of less soluble Cd-As complexes could be the explanation for this reduction. However, soil matrix samples were not included in this study. A few ecotoxicity studies have shown that the phytotoxicity of Cd was mitigated in soils spiked with both As and Cd, which indicates possible interaction between As and Cd in soil matrix can affect the uptake of Cd into plants (Cao et al., 2007; Sun et al., 2008). To the author's knowledge, no study has illustrated whether soil-bearing As and Cd can affect each other's bioaccessibility when they co-occur in simulated human digestive system and moreover, if soil properties could affect this interaction.

There are a number of *in vitro* bioaccessibility testing systems available, among which the Unified BARGE Method (UBM) has been well established and commonly used as a screening tool (Appleton et al., 2012; Barsby et al., 2012). So far the results of UBM have been correlated with bioavailability data of a juvenile swine model (a good representative physiological condition of children) for As, Cd and Pb (Denys et al., 2012). In addition, it is physiological-related by comprising three key cavities (mouth, stomach and intestine) and ingredients of human digestive fluids (e.g. mucin, pepsin, amylase, bile). Moreover, endeavours have been made to assess the reliability of the UBM and to improve between-laboratory variability (Wragg et al., 2011). Even though some uncertainties are still associated with the UBM, it is selected as the indicator of bioavailability in this work.

In this study, seven different types of soil were collected in Australia and spiked with a wide range of concentrations of As or Cd in order to elucidate the ability of soil-metal/metalloid interactions that reflect their respective bioaccessibility. Furthermore, the interaction between As and Cd was studied by mixing soils of the same soil type spiked with As or Cd during the UBM extraction, which mimics human ingestion of mixtures of independently-aged As and Cd in soils. This study helps to understand the interaction between As and Cd in a simulated human digestive system as well as the possible relationship between soil properties and this interaction, which provides new insight into informed risk assessment of mixtures.

2.2 Materials and methods

2.2.1 Sample collection and preparation

Seven chemically-variant top soils (0-20 cm) were collected from Victoria and South Australia in Australia (Figure S1 of Appendix 2). The locations were in the townships of Dublin (DUA), Kersbrook (KBA), Millicent (MIA), Mount Gambier (MGA), Port Broughton (PBA), Tarrington (TAA) and Wallaroo (WRA). Soils were air-dried at room temperature and sieved through 2 mm stainless steel sieves. Soils were stored in plastic containers at room temperature until use.

2.2.2 Soil characterisation

Soil pH was determined potentiometrically (TPS SmartCHEM) at a 1:5 ratio of soil to 10 mmol L^{-1} $CaCl_2$ after 1 h of end-to-end shaking. Elemental analysis of total carbon (TC), nitrogen (TN) and sulphur (TS) was measured by TOC analyser (Leco TruMac CNS analyser). Total organic carbon (TOC) content was determined after HCl addition to eliminate carbonates as recommended by the manufacturer. Cation exchange capacity (CEC) was measured following the compulsive exchange method (Gillman and Sumpter, 1986). Oxalate-extractable iron (Fe), aluminium (Al) and manganese (Mn) concentrations (representative of the amorphous Fe, Al, Mn oxide contents) were determined using the acid oxalate method (pH=3) (Rayment and Higginson, 1992). Sand, clay and silt contents were determined using the hydrometer method (Gee and Bauder, 1986). Dissolved organic carbon (DOC) was determined using a total carbon analyser (1010 OI Analytical).

2.2.3 Soil spiking

After being air-dried, soils were spiked with As (sodium arsenate, $Na_2HAsO_4 \cdot 7H_2O$) and Cd (cadmium nitrate, $Cd(NO_3)_2 \cdot 4H_2O$). Chemicals were purchased from Sigma-Aldrich, Australia. Even though As has various speciation in soils (e.g. arsenite, arsenate, monomethylarsonic acid, dimethylarsinic acid), the pentavalent As (V) is the stable oxidation state in surface soils to which children can have easy access and also reported as the most frequent species found in soils (Garcia-Manyes et al., 2002; Yang et al., 2002). Cadmium speciation is less complex for the reason that Cd is normally found bivalent and the principal species of Cd in the soil solution is free ions and hydrolysed ions. Thus sodium arsenate and cadmium nitrate (soluble in water) were the appropriate compounds to be spiked in soils. During the ageing period and UBM extraction, As and Cd are not likely to undergo species transformation. Chemical solutions were sprayed to a thin layer of soil with continuous mixing using a trowel in a plastic tray. Then spiked soils were placed in plastic containers and mixed again by mechanical mixer for another 5-10 minutes. Soils were incubated at water contents of approximately 60-70% (w/w) of field capacity by periodical replenishment of water during one-year ageing. The control of each soil was prepared and stored in the same manner.

The spiked concentrations of As and Cd in soils were around or above the Australian National Environmental Protection Measure (NEPM) for the Assessment of Site Contaminated health investigation level A (HIL A) for standard residential garden/accessible soil and children's day care centres, kindergartens, preschools and primary schools. The HIL A of As and Cd are set at 100 and 20 mg kg^{-1}, respectively (NEPC, 2013a). The criterion for selecting highest As spiked concentration is 10 times as high as HIL A (Basta and Juhasz, 2014a). But the highest concentration spiked in soil type TAA is 20 times higher than HIL A of As for the reason that soil samples are sufficient to mimic highly-contaminated soil whilst some soil types (PBA, DUA) have lower concentrations. Maximum Cd spiked concentrations are especially high with some exceeding 500 mg kg^{-1}. The reason for these high Cd concentrations is that we aim to bring Cd concentrations to the similar levels of As concentration thus interaction would easily detected if there is any. For example, we are interested in interaction between As (1000 mg/kg) and Cd (100 mg/kg). If there is any interaction, the change in Cd bioaccessibility maybe significant whilst the change in As bioaccessibility is not obvious due to the big concentration gap. Therefore, for the purpose of

interaction study, the maximum Cd spiked concentration is around 20 times as high as HIL A of Cd.

After aged soils were dried and sieved <250 μm, total concentrations of As and Cd in spiked soils were analysed using inductively coupled plasma mass spectrometry (Agilent 7500cs ICP-MS, Agilent Technologies, Japan), following the U.S. EPA 3051A dissolution procedure (EPA, 2007). Quality control/quality assurance was ensured by using two soil standard reference materials (NIST SRM 2710a and 2711a, National Institute of Standards and Technology, Washington, DC), spiked samples, duplicates and reagent blank. Internal standard was used when diluted samples were run on ICP-MS in order to control matrix effect and signal drift. The limit of detection (LOD) was defined three times the standard deviation of blank. The LODs of As, Cd and Pb were <0.1 μg L^{-1} each running.

2.2.4. Bioaccessibility measurement

Spiked soils, after ageing, were dried and sieved to <250 μm for the bioaccessibility measurement. The <250 μm particle size is the fraction that is likely to adhere to children's hands and therefore is ingested via hand-to-mouth activities (Duggan et al., 1985; Rodriguez and Basta, 1999). The method was initially designed for 0.6 g of each soil sample to be extracted in 60 mL UBM simulated solution. However in this study, the procedure was modified for 0.3 g subsample and 30 mL UBM solution. ANOVA result demonstrated no significant difference (p>0.05) was observed between 0.6 g and 0.3 g extractions for As and Cd in NIST SRM 2710a and 2711a (data not shown). A schematic diagram of the UBM methodology, as well as ingredients of UBM digestive fluids, is provided in Appendix 1. For the mixture study, equal portions (0.15 g) of As-spiked soil and Cd-spiked soil were weighed together in the same container and extracted in UBM fluids. After UBM extraction, solutions were filtered through 0.45 μm filter (PVDF, Millipore) and diluted with 2% HNO3 to be measured on ICP-MS. Triplicate samples, reagent blank, spiked samples and standard reference materials (NIST SRM 2710a and 2711a) were used for quality control/quality assurance. Internal standard was used when diluted samples were run on ICP-MS in order to control matrix effect and signal drift. The limit of detection (LOD) was defined three times the standard deviation of blank. The LODs of As, Cd and Pb were <0.1 μg L^{-1} each running.

Throughout the UBM, bioaccessible As or Cd is referred to extracted As/Cd (in mg) from each kg soil sample (dry weight). Bioaccessibility is expressed in percentage (%) that corresponds to the ratio between bioaccessible As or Cd and the total As or Cd concentration in soil (<250 μm). Gastric bioaccessibility is for the worst-case scenario (Makris et al., 2008) whilst intestinal bioaccessibility is physiologically-related where absorption mainly takes place after ingestion.

2.2.5 Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 (La Jolla, USA). Linear regressions were undertaken to analyse the relationship between bioaccessible concentration and total concentration as well as between the bioaccessibility and soil physico-chemical properties (pH, CEC, TOC, Fe oxide, Al oxide etc.). Regression with $r^2 > 0.5$, $p < 0.05$ was considered as significant. Data were compared using unpaired Students' t-test without Welch correction (assuming same variances). Significant level, $p < 0.05$, was adopted to determine whether the means of two compared groups were significantly different.

2.3 Results and discussion

2.3.1 Physicochemical properties of soils

Soil properties are shown in Table 2.1. These soils had a loamy texture with different contents of clay, sand and silt. pH values ranged from 4.45 to 7.73. TC was in the range of 1.6 to 8.37 % without significant amounts of TN and TS detected. TOC varied between 1.48 and 8.37% whilst DOC showed a wide range from 106 to 400 mg kg^{-1}. CEC values ranged from 2.9 to 17.9 cmol kg^{-1}. Concentrations of Fe oxide, Al oxide, and Mn oxide were 0.28-12.49, 0.5-12.6 and 0.0252-0.302 g kg^{-1}, respectively. Total concentrations of As and Cd in control and spiked soil after one-year ageing were listed in Table S1 (Appendix 2). The difference between the spiked concentrations and measured ones may be due to the particle size (spiked soils were sieved to <250 μm), which indicates inappropriateness of some previous studies (Tang et al., 2006c) measuring bioaccessibility without re-analysing total concentrations after spiking, ageing and sieving. Previous studies have also reported the variability of metal/metalloid concentration in different particle sizes, which cast doubt on the thought that concentration of metal/metalloid is homogeneously distributed across different

size fractions of soil (Juhasz et al., 2011a; Beamer et al., 2012). A general observation is that concentrations of contaminants are higher in small particle sizes due to a higher a greater reactive surface area but ultimately depends on the species of binding sites and the distribution of these binding sites in different soil size particles (Ljung et al., 2006). Measured concentrations were used for calculation in this study. Concentrations of Cd in all spiked soils exceeded HIL A (20 mg kg^{-1}) whilst many soils contained As levels higher than HIL A (100 mg kg^{-1}). For the purpose of interaction study, soils containing As concentrations lower than HIL A were also included. Concentrations of As in the SRMs were found to be 1418±105 mg kg^{-1} (certified values: 1540±10 mg kg^{-1}) for NIST 2710a and 90.5±8.5 mg kg^{-1} (certified values: 107±5 mg kg^{-1}) for NIST 2711a (n=6) whilst Cd concentrations were 10.7±0.8 mg kg^{-1} (certified values: 12.3±0.3 mg kg^{-1}) for NIST 2710a and 48.5±4.1 mg kg^{-1} (certified values: 54.1±0.5 mg kg^{-1}) for NIST 2711a (n=6), respectively.

Table 2.1 Soil properties of the seven spiked soils collected in Australia

	MIA	MGA	KBA	TAA	WRA	PBA	DUA
Soil location	Millicent	Mount Gambier	Kersbrook	Tarrington	Wallaroo	Port Broughton	Dublin
pH	7.12	5.68	4.45	4.92	7.66	7.73	7.31
Clay (%)	10	16.3	20	10	17.5	6.7	7.5
Silt (%)	6.3	21.9	37.5	17.5	23.8	10.7	12.8
Sand (%)	83.8	61.9	42.5	72.5	58.8	82.7	79.8
TC (%)	4.11	8.37	5.5	4.97	5.33	2.07	1.6
TN (%)	0.00579	0.0261	0.0152	0.015	0.00598	0.00265	0.00229
TS (%)	0.02	0.01	0	0.01	0	0	0
TOC (%)	3.86	8.37	5.5	4.97	3.54	1.76	1.48
DOC (mg kg^{-1})	303	391	400	326	265	106	213
CEC(cmol kg^{-1})	13	17.9	4.3	7.4	13.7	4.6	2.9
Fe oxide (g kg^{-1})	0.43	12.49	1.72	3.12	0.47	0.28	0.64
Al oxide (g kg^{-1})	2.27	12.6	2.13	3.01	1.38	0.5	0.74
Mn oxide (g kg^{-1})	0.0252	0.302	0.0449	0.212	0.237	0.0595	0.133

Data represent the mean of duplicate analysis. Values varied by less than 5%.

2.3.2 Bioaccessibility of As and Cd in spiked soil

Table 2.2 shows the bioaccessibility of As and Cd which ranged from 40±2.8 to 95±1.3% and 72±4.3 to 99±0.8% for the gastric phase, and from 16±2.0 to 96±0.8% and 6.2±0.3 to 45±2.7% for the intestinal phase, respectively. Moreover, for each soil type, the bioaccessibility of different concentrations of As or Cd were very similar. The high solubility of Cd in the gastric phase indicates, after one-year ageing in soils, Cd can still be easily released from soil-binding sites under acidic environment (pH=1.2-1.5). After soil moved into the intestinal phase, bioaccessible Cd dropped significantly. Possible reasons have been proposed previously such as (1) re-adsorption of Cd on the soil components, (2) complexation by pepsin, and (3) chemical precipitation of elements due to the increase of pH in the intestinal phase (Grøn, 2003; Basta et al., 2005). Only slight difference was observed between As gastric bioaccessibility and As intestinal bioaccessibility except in MGA soil samples for which As intestinal bioaccessibility was half of the value of its gastric bioaccessibility. The particularity of MGA soils is that it contains a relatively higher amount of organic carbon, Fe oxide and Al oxide. Since negatively-charged Fe oxide will release As at elevated pH when soils moved to intestinal phase (Raven et al., 1998; Smedley and Kinniburgh, 2002), the decrease of As intestinal bioaccessibility may be due to the adsorption of As on organic carbon with other metals. For the results of NIST SRM 2710a, the averaged gastric bioaccessible As and Cd were 487±28 mg kg^{-1} and 4.8±0.3 mg kg^{-1} and intestinal bioaccessibility were 412±30 mg kg^{-1} and 2.8±0.33 mg kg^{-1}, respectively (n=6). For NIST 2711a, the bioaccessibility of As and Cd for gastric phase were 46.5±2.7 mg kg^{-1} and 41.6±3.2 mg kg^{-1} and 41.4±5.0 mg kg^{-1} and 13.8±0.7 mg kg^{-1} for intestinal phase (n=6). These results were in agreement with those reported in previous studies (Roussel et al., 2010; Wragg et al., 2011). The consistency of results obtained for the reference materials suggests good reproducibility of UBM.

Table 2.2 Bioaccessibility of As and Cd in seven different types of spiked soils

	As bioaccessibility			Cd bioaccessibility		
Soil code	Total As (mg kg^{-1})[a]	Gastric phase (%)[b]	Gastric + intestinal phase (%)[b]	Total Cd (mg kg^{-1})[a]	Gastric phase (%)[b]	Gastric + intestinal phase (%)[b]
MIA	69	84±1.5	83±2.9	20	99±0.8	32±0.7
	200	74±1.6	76±7.6	51	93±6.8	23±1.3
	732	85±0.2	74±6.2	174	90±0.6	26±0.7
				332	80±8.2	31±1.4
MGA	128	41±1.9	16±2.0	38	86±1.9	6.3±0.4
	313	43±2.7	18±6.5	102	87±2.4	6.2±0.3
				185	93±4.5	8.1±0.5
				380	91±1.7	10.2±0.2
				833	94±3.8	12±0.3
KBA	80	68±3.4	68±2.8	26	89±3.2	24±0.04
	145	63±6.1	70±6.9	61	74±1.6	20±4.5
	402	60±0.6	57±4.1	91	82±1.5	23±0.5
	1051	68±1.5	60±11	261	87±2.7	28±0.4
				671	88±1.5	29±0.7
TAA	76	43±0.8	43±0.9	30	90±18	22±2.8
	206	40±2.8	41±2.1	63	78±2.9	17±1.9
	455	42±8.3	38±12	110	84±3.5	20±0.6
	903	55±4.5	47±11	306	88±2.0	19±4.3
	1930	55±0.7	40±1.9	775	90±0.5	25±0.6
WRA	80	94±0.5	89±0.6	50	94±5.9	31±4.4
	211	91±3.8	83±3.2	95	73±3.5	27±3.2
	522	76±2.3	69±2.2	258	74±5.6	28±0.5
				570	82±8.9	33±5.4
PBA	86	95±1.3	96±0.8	41	72±4.3	30±2.0
	247	81±4.2	80±3.7	152	90±3.2	44±1.3
				338	88±2.1	45±2.7
DUA	101	83±9.3	73±11.2	40	93±1.1	41±0.5
	248	79±5.5	80±2.8	75	90±5.7	41±0.6
				218	85±6.4	44±0.4
				413	85±12	44±3.1

[a] Data represent the mean of measured concentrations (same as shown in Table S1 in Appendix 2).
[b] Data represent the mean of triplicate measurements ± standard deviation (SD).
The <250 μm particle size was used for all measurements.

2.3.3 Relationships between the bioaccessibility, total concentrations and selected soil properties

Strong linear relationships between bioaccessible As/Cd (both gastric and intestinal phases) and total As/Cd in soils were observed with r^2 varying between 0.72 and 0.98 (Figure 2.1). Therefore, within the spiked concentrations of As and Cd in this study, the soluble As and Cd during UBM extraction increased proportionally along with total concentrations in soils. Relations in gastric phase were stronger than that in intestinal phase probably due to the high solubility of both As and Cd in the gastric phase. These bioaccessibility results were consistent with previous field studies (Juhasz et al., 2007b; Sarkar et al., 2007; Meunier et al., 2010; Roussel et al., 2010; Das et al., 2013), which indicate As and Cd in spiked soil acted similarly as field soils to some extent.

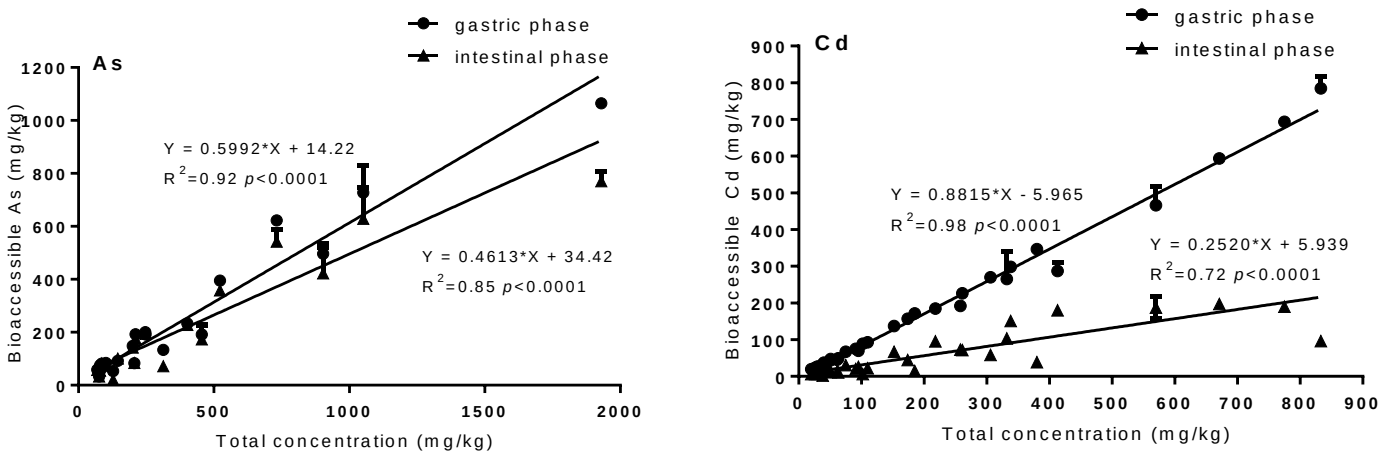

Figure 2.1 Linear regression analysis between bioaccessible As/Cd and total As/Cd concentrations in spiked soils. Each data is mean ± standard deviation (SD), n=21 (As), n=30 (Cd).

Soil physicochemical properties including TOC, pH, clay, Fe oxide, Al oxide and Mn oxide were selected to explore key soil parameters which can affect bioaccessibility (Table 2.3, Figures S2-S3). Organic carbon, Fe oxide and Al oxide were three predominant soil properties controlling the physiological-related bioaccessibility of As and Cd, among which soil organic carbon was the most influential one. This result is within expectation as organic amendment has long been adopted as an effective tool for the remediation of heavy metal/metalloids in soils (Park et al., 2011). Furthermore, the affinity of Cd on organic matter was found stronger than that of As (reflecting on the r^2 values), which is in accordance with the general order of affinity of heavy metal/metalloids on organic matter (Takamatsu et al., 1983; Warwick et al., 2005). Moreover, dissolved organic carbon played a more important role in controlling Cd bioaccessibility ($r^2 = 0.61$). Park et al. summarised that Fe oxide and Al oxide can act either as a sorbent or as precipitating agent to reduce the bioavailability of metal/metalloids, which is also demonstrated in this study (Park et al., 2011). In addition, positive relationship between As bioaccessibility and pH was observed because the adsorption of negatively-charged As on Fe oxide can be enhanced under acidic conditions, where Fe oxide is mainly positively charged (Yang et al., 2002; Juhasz et al., 2007b; Das et al., 2013). One unexpected result is that there was no relationship between the bioaccessibility and clay content whilst an increase in clay was reported to decrease the bioaccessibility of As in several studies (Sarkar et al., 2007; Girouard and Zagury, 2009; Das et al., 2013). In our case, without natural weathering, As and Cd may form outer-sphere complex with clay rather than inner-sphere complex and this complexation can be easily disrupted under both gastric and intestinal conditions (Goldberg, 2002).

One limitation of these relationships is that soils with high content of Fe oxide contain high Al oxide as well thus the effect of Fe oxide cannot be differentiated from that of Al oxide. Further study could be performed to identify the roles that Fe oxide and Al oxide may play in controlling the bioaccessibility of As and Cd by adopting soils containing similar/different contents of Fe oxide and Al oxide. Another limitation is that relationships were extrapolated using limited soil types (7 in this study). Therefore, interpretation of soil-bioaccessibility relationships based on values of r^2 (goodness of fit) should be taken with caution. Inclusion of more soil types would help to avoid any incidental correlation between soil properties as well as to strengthen the conclusion of the current work.

The variability of relationships between bioaccessibility and soil properties has been reported in numerous studies, which exemplifies bioaccessibility is controlled by a number of factors such as speciation, mineralogy, duration of contaminant-soil contact time and even extraction methods (Oomen et al., 2002; Reeder et al., 2006; Tang et al., 2007b; Meunier et al., 2010). In the current study, total organic carbon, Fe oxide and Al oxide were found to be the key soil properties governing bioaccessibility. These correlations were explored with an aim to identify whether the key parameters controlling bioaccessibility are important for the possible interaction between As and Cd during UBM extraction.

Table 2.3 Relationships (goodness of fit, r^2) between bioaccessibility and selected soil properties

Soil property	As gastric bioaccessibility		As intestinal bioaccessibility		Cd gastric bioaccessibility		Cd intestinal bioaccessibility	
	r^2	*p*	r^2	*p*	r2	*p*	r^2	*p*
pH	0.57 (+)	<0.0001	0.43	<0.0001	0.005	0.10	0.4	<0.0001
Clay (%)	0.024	0.17	0.025	0.07	0.028	0.68	0.27	<0.0001
Silt (%)	0.062	0.04	0.046	0.07	0.000085	0.93	0.12	0.0005
Sand (%)	0.052	0.06	0.05	0.06	0.00009	0.93	0.25	<0.0001
TC (%)	0.41	<0.0001	0.55(-)	<0.0001	0.037	0.013	0.83(-)	<0.0001
TOC (%)	0.58 (-)	<0.0001	0.7 (-)	<0.0001	0.048	0.001	0.85(-)	<0.0001
DOC (mg kg-1)	0.37	<0.0001	0.4	<0.0001	0.023	0.001	0.61(-)	<0.0001
CEC (cmol kg-1)	0.004	0.6	0.088	0.56	0.025	0.02	0.45	<0.0001
Fe oxide (g kg-1)	0.48	<0.0001	0.65 (-)	<0.0001	0.08	0.003	0.6(-)	<0.0001
Al oxide (g kg-1)	0.39	<0.0001	0.59 (-)	<0.0001	0.1	0.002	0.63(-)	<0.0001
Mn oxide (g kg-1)	0.16	<0.0001	0.26	<0.0001	0.035	0.52	0.33	<0.0001

"G" represents gastric phase; "GI" represents intestinal phase. "+" means positive relationship; "-" means negative relationship. Highlighted values represent strong relationships ($r^2 > 0.5$, $p<0.0001$) between soil property and bioaccessibility. Since TOC, which varied from 1.48 to 8.37%, was the dominant component of TC in these seven types of soils, r^2 between TC and bioaccessibility was not highlighted. "n" used in the regression model is 7 (seven types of soils) for each soil property and As/Cd bioaccessibility.

2.3.4 Interaction between As and Cd during UBM extraction

Table 2.4 summarises the results of the interaction between As and Cd during UBM extraction. Statistical comparison shows the interaction was not pronounced (p>0.05) in any of the seven soil types (Table S2 in Appendix 2). Explanation could be that there were plenty of combining sites (e.g. organic carbon, Fe oxide, Al oxide) for both As and Cd in their separate spiked soils thereby As and Cd aged independently. When interaction study was undertaken by mixing two separate portions of the same type of soil spiked with As or Cd, As and Cd would more likely to stay with their own binding agency, showing no effect on respective bioaccessibility. In addition, concentrations of free Cd^{2+} and AsO_4^{3-} were decreased to a significant extent due to the presence of thiol groups of proteins (e.g. bovine serum albumin, bile). Thus the formation of less soluble $Cd_3(AsO_4)_2$ did not seem to happen during UBM test as it was reported in a rat bioavailability study (dose pure solution) and a phytotoxicity study (Cao et al., 2007; Diacomanolis et al., 2014). This result indicates the bioaccessibility of As and Cd could be calculated separately under real scenario where children ingest soil containing independently-aged As and Cd, e.g. there is a long time gap between As and Cd entering the same spot or children take in As-containing soil at one spot followed by ingestion of Cd-containing soil at another spot. However, additional future work will enable this assumption more convincing. Although UBM is physiological-based, it is an *in vitro* simulation of the human digestive environment**.** *In vivo* experiment would help to add robust evidence to this assumption by dosing animals with soil containing As or Cd simultaneously. On the other hand, how As and Cd are spiked may play an important role in the possible interaction. In this study, As and Cd were spiked separately and underwent independent ageing process. But there is another scenario where As and Cd enter the same soil spot simultaneously and age together, which can be mimicked by sequentially spiking As and Cd in the same soil. This kind of work will be further discussed in Chapter 4, which will gain additional information about mixed contaminants.

Table 2.4 The interaction between As and Cd during UBM extraction

	As bioaccessibility			Cd bioaccessibility		
Soil code	Total As (mg kg^{-1})[a]	Gastric phase (%)[b]	Gastric + intestinal phase (%)[b]	Total Cd (mg kg^{-1})[a]	Gastric phase (%)[b]	Gastric + intestinal phase (%)[b]
MIA	69	84±1.5	83±2.9	51	93±6.8	23±1.3
	69 (51)	82±0.2	82±2.2	51 (69)	94±5.5	23±1.0
	732	85±0.2	74±6.2	332	80±8.2	31±1.4
	732 (332)	88±1.7	80±1.4	332 (732)	83±12	32±5.5
MGA	128	41±1.9	16±2.0	102	87±2.4	6.2±0.3
	128 (102)	35±2.7	18±1.9	102 (128)	94±5.4	6.5±0.5
	313	43±2.7	18±6.5	833	94±3.8	12±0.3
	313 (833)	38±0.5	12±2.6	833 (313)	90±4.5	10.5±0.1
KBA	80	68±3.4	68±2.8	61	74±1.6	20±4.5
	80 (61)	62±0.8	62±0.8	61 (80)	73±0.5	16±0.5
	1051	68±1.5	60±11	671	88±1.5	29±0.7
	1051 (671)	69±9.7	68±3.7	671 (1051)	76±2.4	25±0.7
TAA	76	43±0.8	43±0.9	63	78±2.9	17±1.9
	76 (63)	36±0.9	40±1.2	63(76)	82±3.7	16±0.9
	903	55±4.5	47±11	775	90±0.5	25±0.6
	903 (775)	51±2.3	50±5.3	775 (903)	84±1.4	21±1.4
WRA	80	94±0.5	89±0.6	95	94±5.9	31±4.4
	80 (50)	89±1.5	85±1.5	95 (80)	97±2.1	34±0.7
	522	76±2.3	69±2.2	570	82±8.9	33±5.4
	522 (570)	73±9.1	69±2.3	570 (522)	92±8	33±4.3
PBA	86	95±1.3	96±0.8	41	72±4.3	30±2.0
	86 (41)	92±2.2	94±1.1	41 (86)	72±1.3	31±0.2
	247	81±4.2	80±3.7	338	88±2.1	45±2.7
	247(338)	84±5.4	81±2.4	338 (248)	88±1.1	39±2.1
DUA	101	83±9.3	73±11.2	40	93±1.1	41±0.5
	101 (40)	78±3.1	81±3.6	40 (101)	94±2.1	40±0.8
	248	79±5.5	80±2.8	413	85±12	44±3.1
	248 (413)	85±2.5	78±4.3	413 (248)	85±1.9	43±0.5

"As bioaccessibility", 69(51) means 0.15 g soil spiked with 69 mg kg^{-1} As was mixed with 0.15 g soil spiked with 51 mg kg^{-1} Cd, etc; "Cd bioaccessibility", 51(69) means 0.15 g soil spiked with 51 mg kg^{-1} Cd was mixed with 0.15 g soil spiked with 69 mg kg^{-1} As, etc.

[a] Data represent the mean of duplicate measurements.

[b] Data represent the mean of triplicate measurements ± standard deviation (SD).

The <250 μm particle size was used for all measurements.

2.4 Conclusion

This study has identified strong relationships between bioaccessible concentrations of As/Cd and total As/Cd concentrations in soils. Organic carbon, Fe oxide and Al oxide were found to be key soil properties influencing As bioaccessibility and Cd intestinal bioaccessibility but these relationships need further validation by including more soil types in the future. These key soil properties were not important for the interaction between As and Cd during UBM extraction. It shows the interaction between As and Cd was not apparent in any tested soils, which suggests within the concentration scope, spiking and ageing process in this study, As and Cd may act independently after being ingested in human gut simultaneously. These findings have significant implications for the understanding of the bioaccessibility of mixtures. Also, this research has raised a number of questions in need of further investigation, such as different spiking procedures, more complex mixtures, which will be addressed in the latter chapters.

Chapter 3 Effects of arsenic and cadmium on bioaccessibility of lead in spiked soils assessed by Unified BARGE Method

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Abstract

The bioaccessibility of Pb in contaminated soils has been extensively studied, including the influence of soil properties on Pb bioaccessibility. However, little is known about the effects of other metal/metalloid, such as As, Cd on the bioaccessibility of Pb, i.e. whether As or Cd could increase or decrease the solubility of Pb in human gastrointestinal tract when Pb-contaminated soil and As-contaminated (or Cd-contaminated) soil are ingested simultaneously. Furthermore, it is far from clear that if soil property could have any effect on this possible interaction. In this study, seven types of soils were collected in Australia and spiked with As, Cd or Pb. Gastric bioaccessibility of Pb ranged from 44±0.9% to 100±6.7% whilst intestinal bioaccessibility dropped to 1±0.2% to 36±1.7%. Statistical analysis shows total Pb in soil was the most significant controller for bioaccessible Pb. Effects of As and Cd on the bioaccessibility of Pb in simulated human digestive system were studied by mixing As-spiked soil (or Cd-spiked soil) with Pb-spiked soil of the same type in the same container during bioaccessibility test. Results reveal that neither As nor Cd had an impact on Pb bioaccessibility and vice versa, which indicates when As, Cd and Pb aged in soils separately, they may behave independently in the bioaccessibility measuring system. Combining the results in Chapter 2, this finding can be part of the evidence to assume additive effect when it comes to estimating the bioaccessibility of mixtures of independently-aged As, Cd and Pb in soils.

3.1 Introduction

Lead (Pb), as a non-essential element for human, has long been a public concern due to its significant build-up in the environment and well-known toxicity. For the past three centuries, the contamination level of Pb in the environment has been elevated three orders of magnitude as a result of human activities in the production of Pb and its utilisation, such as coal burning, mining, smelting, Pb-containing paint, leaded gasoline, pesticide, waste incineration (ATSDR, 2007b). Toxicological and epidemiological studies indicate Pb has the potential to cause systemic effects, neurological effects, immunological and lymphoreticular effects, reproductive effects, developmental effects and deaths (ATSDR, 2007b). Even of more concerned, Pb poisoning gives rise to frequent and severe environmental disease affecting the health of young children (Ryan et al., 2004). Extensive evidence has shown that high Pb in blood inhibits the cognitive development of children (Dietrich et al., 1991; Lanphear et al., 2000). Young children can be exposed to Pb via drinking water, food, paint and soil, of which inadvertent ingestion of soil is a major route due to their hand-to-mouth activities, especially for children with pica behaviour who may ingest up to 50 g soil per day (Calabrese et al., 1989; Calabrese et al., 1999). Since Pb usually stays in the upper layers of soil (Khan and Frankland, 1983), it increases the possibility for children to ingest Pb-bearing soils. Elevated levels of blood Pb in children who lived near Pb-polluted sites have been widely reported (Landrigan and Baker, 1981; Duggan and Inskip, 1985; Hilts et al., 1998). Once exposure risk is identified, remediation program should be initiated in order to safeguard the most sensitive population. It is widely accepted that bioavailability and bioaccessibility should be considered when making remediation decisions since not 100% Pb can be absorbed into the human circulatory system unless the bioavailability is 100% (Berti and Cunningham, 1997; Hettiarachchi and Pierzynski, 2004; Bosso et al., 2008). In Australia, when Pb concentration in soil is found to be above Health Investigation Level of National Environment Protection (Assessment of Site Contamination) Measure, bioaccessibility, which represents the soluble part of a contaminant in human gastrointestinal tract, is also recommended as a tier-two risk assessment (NEPC 2013). Bioavailability data can be obtained by using *in vivo* models as reviewed (Ng et al., 2015). However, animal experiments are costly, time-consuming and associated with ethical problems thus several *in vitro* bioaccessibility models have been used as a surrogate for bioavailability (Ng et al., 2015). These *in vitro* methods which have been correlated with bioavailability data measure the solubility of metal/metalloids in simulated human digestive fluids.

To date, *in vivo* bioavailability and *in vitro* bioaccessibility models have been mostly based on the performance of Pb alone without considering the existence of other metal/metalloid that could influence its bioavailability or bioaccessibility. Soils are frequently co-contaminated with a range of metals/metalloid, such as As, Cd, Cr, Cu, Ni, Zn, among which As and Cd attract significant attention due to their ubiquity and toxicity (ATSDR, 2004, 2007a). Phytotoxicity studies demonstrate the interaction between Pb and Cd with regard to plant growth (Hassett et al., 1976; Khan and Frankland, 1983). It is also reported that oral bioaccessible Pb was increased along with the increase in total Cd concentration in soil (Pelfrene et al., 2011). Lead and As commonly co-exist at contaminated sites as a result of lead arsenate ($PbHAsO_4$) being used as pesticide in orchids and their co-occurrence in mineralised ore materials (Arai et al., 2006; Vaca-Escobar et al., 2012). Lead arsenate is highly likely to be dissociated into ionic species in the acidic gastric phase as arsenate is soluble in acid, but exists as an insoluble compound in the alkaline conditions of the intestinal phase. Besides, water-extractable As was reported significant for controlling bioaccessible Pb in gastric and small intestinal phases (Cui and Chen, 2011). To our knowledge, there is no detailed investigation to illustrate whether As or Cd can influence the bioaccessibility of Pb when soils containing mixture of As and Pb (or Cd and Pb) are ingested into human digestive system.

Except for possible effects that As or Cd may exert on Pb bioaccessibility, a variety of soil parameters (pH, phosphate, iron oxides, organic matter, etc) could be key controllers for Pb bioaccessibility (Zia et al., 2011). However, these controllers need to be applied on a site-specific case. Moreover, it is unclear that whether soil properties could make a difference to the effects of As or Cd on Pb bioaccessibility. Thus in this study, seven types of soils with a wide range of soil properties were spiked with different levels of As, Cd or Pb individually. For the mixture interaction studies, Pb and As-spiked (or Cd-spiked) soils were loaded together in the bioaccessibility extraction system, which mimics conditions of the human digestion system containing mixtures of Pb and As (or Pb and Cd). This study helps to understand the impact of As and Cd on the bioaccessibility of Pb during *in vitro* bioaccessibility extraction and the role that soil properties may play on this possible interaction. The Unified BARGE Method (UBM) is selected for bioaccessibility measurement in this study for the reasons stated in the introduction of Chapter 2. This study is a follow-up work of Chapter 2 in which the interaction between As and Cd during UBM

extraction was investigated and would provide further valuable information for risk assessment of mixed contaminants including Pb and As or Pb and Cd.

3.2 Materials and methods

3.2.1 Soil sampling and characterisation

Seven variant soils were collected from Victoria and South Australia, namely Dublin (DUA), Kersbrook (KBA), Millicent (MIA), Mount Gambier (MGA), Port Broughton (PBA), Tarrington (TAA) and Wallaroo (WRA). Details are available in Section 2.2.1 and 2.2.2 (page 45) regarding soil processing, storage as well as the analysis for pH, total carbon (TC), total nitrogen (TN), total sulphur (TS), total organic carbon (TOC), cation exchange capacity (CEC), oxalate-extractable iron (Fe), aluminium (Al) and manganese (Mn) concentrations (representative of the amorphous Fe, Al and Mn oxide contents) and particle size distribution. Excepting for these parameters, more properties were reported in this chapter: soil textures were categorised according to the U.S. soil classification system (USDA, 1987); electrical conductivity (EC) was measured according to a standard protocol (Rayment, 1992); total Fe, Al and Mn were measured by inductively coupled plasma optical emission spectrometry (Optima 8300 ICP-OES, PerkinElmer, U.S.). Total P in seven types of soils measured by carbonate fusion method via Flow Injection Analysis.

3.2.2 Soil spiking

After being air-dried, seven types of soils were spiked with As (sodium arsenate, $Na_2HAsO_4 \cdot 7H_2O$), Cd (cadmium nitrate, $Cd(NO_3)_2 \cdot 4H_2O$) or Pb (lead nitrate, $Pb(NO_3)_2$), individually. These chemicals were purchased from Sigma-Aldrich, Australia. Spiking procedure and soil maintenance were described in Section 2.2.3 (pages 46-47). Soils were aged for one year at ambient temperature. There is no literature to suggest that As, Cd and Pb may undergo change of valence during the ageing period and UBM extraction. Spiked concentrations of As, Cd and Pb were based on the Australian National Environmental Protection Measure (NEPM) for the Assessment of Site Contamination health investigation level A (HIL A) for standard residential garden/accessible soil and children's day care centres, kindergartens, preschools and primary schools. The HILs A for As, Cd and Pb are 100, 20 and 300 mg kg^{-1}, respectively (NEPC, 2013a).

3.2.3 Bioaccessibility measurement

After ageing for one year at ambient temperature, control and spiked soils were dried under 40 ℃ and sieved <250 μm. Prior to bioaccessibility measurement, total concentrations of As, Cd and Pb in sieved soils were determined. The UBM procedure (Appendix 1) was modified for 0.3 g subsample and 30 mL solution for sample conservation. Students' t-test result demonstrated no significant difference (p>0.05) was observed between 0.6 g and 0.3 g of soil used for the extraction for Pb (data not shown). For the mixture study, equal portions (0.15 g) of Pb-spiked soil and As-spiked (or Cd-spiked) soil were weighed sequentially into the same container and extracted in UBM fluids. Throughout this study, bioaccessible Pb is referred to the extracted Pb (in mg) from each kg soil sample (dry weight). Bioaccessibility which is expressed in percentage (%) refers to the ratio between bioaccessible Pb and total Pb concentration in soil (<250 μm).

3.2.4 Quality assurance (QA) and quality control (QC)

In order to test the accuracy and precision of digestion procedure for spiked concentrations (<250 μm) and the consistency of UBM, two soil standard reference materials (NIST SRM 2710a and 2711a) were utilised in this study. Results of reference materials are summarised in Table S1 (Appendix 3). The precision can be manifested by the standard deviation (SD) of these data whilst comparing with certified or published data evaluates the accuracy or consistency of measurement. Blank control and SRMs were run every ten samples. Also, duplicate (total concentration measurement), at least triplicate (UBM extraction) samples and spiked samples were included. Concentrations of As, Cd and Pb were measured by ICP-MS. Internal standard was used when diluted samples were run on ICP-MS in order to control matrix effect and signal drift. The limit of detection (LOD) was defined three times the standard deviation of blank. The LODs of As, Cd and Pb were <0.1 μg L^{-1} each running.

3.2.5 Statistical analysis

Statistical analysis methods regarding linear regression and comparison were described in Section 2.2.5 of Chapter 2 (page 48). In addition, correlation efficiency (p<0.05) between different soil properties was calculated based on Pearson Correlation.

3.3 Results and discussion

3.3.1 Soil characterisation

Table 3.1 summarises soil physio-chemical properties, including those reported in Chapter 2. For soil properties which were not discussed in Chapter 2, discussion were as follows. Soil textures were categorised into loam, loamy sand, sandy loam according to the U.S. soil classification system (USDA, 1987). There was a strong positive correlation between clay and silt (p<0.01) and a negative correlation between clay and sand (p<0.01) as well as silt and sand (p<0.01). An increasing tendency of total carbon was observed with the increase of contents of clay. Total organic carbon (TOC), which varied from 1.48 to 8.37%, was the dominant component of total carbon (TC) except WRA soil showing 1.79% total inorganic carbon (TIC). Electrical conductivity (EC) ranged from 85.8 to 449.5 μs cm^{-1}. Total Fe, Al and Mn were 8.55 to 43.90, 7.98 to 56.64 and 0.06 to 0.50 g kg^{-1}, respectively. It has been demonstrated that metal/metalloid concentrations varied significantly between different particle sizes (Juhasz et al., 2011a; Beamer et al., 2012), which was also confirmed in this study. Total As, Cd and Pb concentrations in sieved <250 μm soils were different from (higher or lower than) theoretical spiked concentrations in <2 mm soils (Table S2 in Appendix 3). Most concentrations of As, Cd and Pb in laboratory soils were above HILs A for As (100 mg kg^{-1}), Cd (20 mg kg^{-1}) and Pb (300 mg kg^{-1}).

Table 3.1 Soil location, soil texture and chemical parameters of seven types of soils

Soil code	MIA	MGA	KBA	TAA	WRA	PBA	DUA
Soil location	Millicent	Mount Gambier	Kersbrook	Tarrington	Wallaroo	Port Broughton	Dublin
pH	7.12	5.68	4.45	4.92	7.66	7.73	7.31
Clay (%)	10	16.3	20	10	17.5	6.7	7.5
Silt (%)	6.3	21.9	37.5	17.5	23.8	10.7	12.8
Sand (%)	83.8	61.9	42.5	72.5	58.8	82.7	79.8
USDA texture*	Loamy sand	Sandy loam	Loam	Sandy loam	Loamy sand	loamy sand	loamy sand
EC (μs cm^{-1})	449.5	128	85.8	124	207.5	236.5	393.5
TC (%)	4.11	8.37	5.5	4.97	5.33	2.07	1.6
TN (%)	0.00579	0.0261	0.0152	0.015	0.00598	0.00265	0.00229
TS (%)	0.02	0.01	0	0.01	0	0	0
TOC (%)	3.86	8.37	5.5	4.97	3.54	1.76	1.48
TIC (%)	0.25	0	0	0	1.79	0.31	0.12
DOC (mg kg^{-1})	303	391	400	326	265	106	213
CEC(cmol kg^{-1})	13	17.9	4.3	7.4	13.7	4.6	2.9
Fe oxide (g kg^{-1})	0.43	12.49	1.72	3.12	0.47	0.28	0.64
Al oxide (g kg^{-1})	2.27	12.6	2.13	3.01	1.38	0.5	0.74
Mn oxide (g kg^{-1})	0.0252	0.302	0.0449	0.212	0.237	0.0595	0.133
Total Fe (g kg^{-1})	18.31	30.95	22.65	43.90	16.80	8.55	9.87
Total Al (g kg^{-1})	33.07	26.99	11.13	56.64	28.31	12.01	7.98
Total Mn (g kg^{-1})	0.07	0.50	0.06	0.27	0.37	0.09	0.17
Total P (g kg^{-1})	0.313	1.35	0.177	0.396	0.338	0.166	0.185

* USDA, the United States Department of Agriculture soil classification; Data represent the mean of duplicate analysis. Values varied by less than 5%.

3.3.2 Bioaccessibility of Pb in spiked soils

Table 3.2 presents the bioaccessibility of Pb in laboratory soils assessed by UBM. For the seven types of soils, gastric Pb bioaccessibility ranged from 44±0.9% to 100±6.7% whilst a pronounced decrease in bioaccessibility (1±0.2% to 36±1.7%) was observed in the intestinal phase. During one-year ageing, Pb may bind to ligands in soils, such as metal oxides, organic carbon, phosphate, and carbonate. However, these Pb-soil complex can be dismissed to a large extent with gastric simulation where pH is 1.2-1.5 causes their hydrolysis, resulting in high bioaccessibility (Pelfrene et al., 2011). Afterwards, the solubilised Pb moved to the intestinal compartment where re-adsorption of Pb on soil ligands, chemical precipitation and

complexation by pepsin may take place, thereby reducing Pb bioaccessibility (Grøn, 2003; Basta et al., 2005).

Interestingly, WRA soil samples showed close to 100% gastric bioaccessibility and extremely low intestinal bioaccessibility (1-2%). It is noticed that, after simulated gastric fluid was added to soils, pH values of all soil suspensions were in the range of 1.2 to 1.5 except for the WRA soil suspension, which showed pH in the range of 2.5 to 3.2 (WRA soil suspension was later adjusted to 1.2-1.5 with 37% HCl). Since the release of gas was observed when adding the gastric solution (pH 0.9-1.0) into WRA soils, it was probably due to the existence of carbonate in WRA soils. Further contact with our collaborator who provided this soil sample confirmed that WRA soils were sampled from an area well known to have substantial calcareous materials. Calcareous soils are mostly or partly composed of calcium carbonate. The following two pieces of evidence indicate a significant amount of calcium carbonate may exist in WRA soils. Firstly, as shown in Table 3.1, total inorganic carbon (TIC), which is largely found in carbonate minerals (Nelson and Sommers, 1996), was 1.79% in WRA soils, significantly higher than those (0 to 0.31%) in other types of soils. Secondly, soil total carbonate is expressed in the form of $CaCO_3$ (calcium carbonate) thus total Ca maybe an indicator of $CaCO_3$. Total Ca in WRA soils was 5 to 30 times as much as those in others (Table S3 in Appendix 3). Taken together, WRA soil was probably calcareous soil and Pb carbonate could be the dominant species in spiked and aged WRA soil samples. This Pb species can be easily solubilised (close to 100% bioaccessibility) under gastric condition (Ruby et al., 1999) and soluble Pb may form precipitates with carbonate, sulphate, phosphate or other anions in alkaline intestinal phase (1-2% bioaccessibility).

Table 3.2 Bioaccessibility of Pb in gastric and intestinal phases in seven types of soils

Soil code	Pb (mg kg^{-1})[a]	Gastric bioaccessibility (%)[b]	Intestinal bioaccessibility (%)[b]
MIA	680	55±1.4	13±0.7
	1047	64±1.6	11±0.5
	3367	89±1.8	16±1.6
	5949	99±2.4	31±3.1
MGA	453	48±0.3	8±2.0
	1139	44±0.9	5±0.3
	2636	65±0.7	8±0.8
	7028	65±6.3	9±3.9
	12225	71±1.3	15±1.9
KBA	569	69±1.8	18±2.5
	1189	86±0.4	24±1.1
	2224	91±0.5	19±0.4
	4544	91±9.3	24±3.6
TAA	309	74±2.4	18±0.6
	756	58±0.7	16±0.4
	1436	61±2.6	15±0.4
	3997	74±2.6	16±3.2
	9345	79±2.8	20±2.9
WRA	289	92±3.2	2±0.9
	1049	100±6.7	2±1.3
	2219	95±9.8	1±0.2
	3898	99±0.3	2±0.2
PBA	445	71±6.2	15±3.0
	864	72±1.5	18±1.8
	2332	74±0.3	18±0.4
	3971	90±7.1	18±0.4
DUA	443	78±12.9	35±1.7
	907	81±0.4	34±0.7
	2495	90±0.4	36±1.7
	4361	93±7.0	29±6.3

[a] Data represent the mean of measured concentrations (same as shown in Table S2, Appendix 3).

[b] Data represent the mean of at least triplicate measurements ± standard deviation (SD).

The <250 μm particle size was used for all measurements.

3.3.3 Relationships between Pb bioaccessibility, total Pb concentration and selected soil properties

Strong linear relationships were observed between bioaccessible Pb (both gastric and intestinal phases) and total Pb concentrations (Figure 3.1), which was in accordance with previous studies (Roussel et al., 2010; Appleton et al., 2012; Reis et al., 2014). Goodness of fit between gastric bioaccessible Pb and total Pb in soil (r^2=0.96, p<0.0001, n=30) was stronger than that between intestinal bioaccessible Pb and total Pb (r^2=0.73, p<0.0001, n=30). Results of WRA soil (1-2%) and soils containing Pb over 5000 mg kg^{-1} were the main reason for lower r^2 value in the intestinal phase. Previously, researchers have pointed out an inflexion point at about 2000 mg kg^{-1} in the regression model correlating total Pb concentration with bioaccessible Pb, which indicates the distinct behaviour of soil Pb at high concentrations (Appleton et al., 2012). Also, the variation associated with intestinal data (shown as error bar in Figure 3.1) was more pronounced than that of gastric data, which was probably due to wider range of pH (5.8-6.5) in the intestinal phase or inhomogeneity and complexation of intestinal solutions (Wragg et al., 2011).

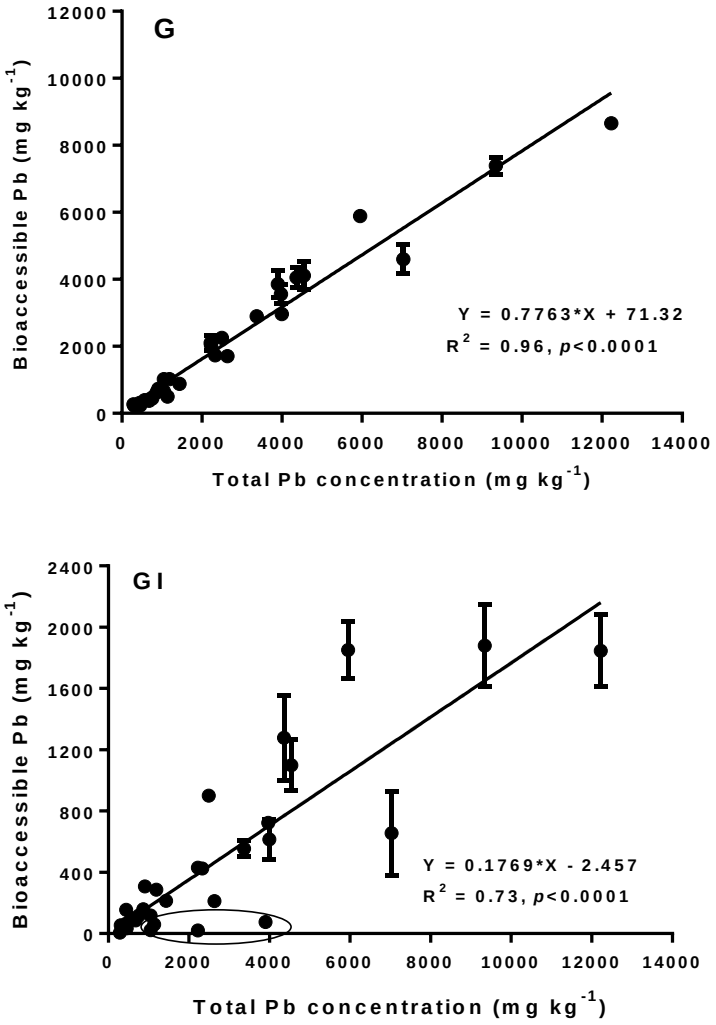

Figure 3.1 Linear regression analysis between bioaccessible Pb and total Pb concentration in spiked soils (n=30). Each data is mean ± standard deviation (SD); "G" means gastric phase, "GI" means intestinal phase; r represents goodness of fit (p<0.0001); Data in the circle are results of WRA soil; Data without error bar means SD is too small to show on the figure.

Linear regression was also carried out between Pb bioaccessibility and selected soil physio-chemical parameters. As shown in Table 3.3 and Figure S1, none of these relationships were statistically significant except for that between Pb intestinal bioaccessibility and CEC (r^2=0.54, p<0.0001, n=7). One reason for the poor relationship between Pb bioaccessibility in intestinal phase and soil properties could be the inclusion of WRA soil samples since total carbonate was believed to be a paramount controller of Pb bioaccessibility in this soil and extremely low intestinal bioaccessibility (1-2%) led to significant deviation of linear regression model. Therefore, linear regression was performed for the second time without the

WRA soil data. It turns out that the goodness of fit between Pb intestinal bioaccessibility and TOC/Fe oxide/Al oxide were dramatically increased to 0.45, 0.41 and 0.43 (p<0.0001, n=7), respectively. These three soil properties also have been reported as crucial parameters for As bioaccessibility and Cd intestinal bioaccessibility with r^2 value ranging from 0.59 to 0.85 (p<0.0001, n=7) in Chapter 2. Since r^2 values of 0.45, 0.41, and 0.43 are not considered as significant, there may be other soil parameters which are important for Pb bioaccessibility.

In the literature, phosphate is a widely-reported key factor that reduces Pb bioaccessibility by forming insoluble minerals such as $Pb_5(PO_4)_3X$ where X includes F, Cl, Br or OH (Bosso et al., 2008; Pelfrêne et al., 2012). An inverse relationship trend between Pb bioaccessibility and total P was observed (Figure S2 in Appendix 3) although it was not significant (p>0.05). The insignificant relationship is probably due to limited number of samples tested and low range of total P in this study. Even though simulated UBM fluids contain phosphate salts (i.e. NaH_2PO_4, KH_2PO_4), $H_2PO_4^-$ is the main species which does not form a precipitate with Pb thus affecting the bioaccessibility. In addition, strong relationship between bioaccessible Pb and total Pb in soil (Figure 3.1) indicates phosphate salts in simulated solution may not precipitate Pb significantly. Current study put emphasis on soil property's effects but carbonate and phosphate soils should be included in future studies to elucidate mineral effect on Pb bioaccessibility.

Throughout the literature, there was a lack of consistency in the relationships between Pb bioaccessibility and soil properties, which indicates that these relationships could be site-dependent and subject to the influence of a variety of other factors, such as speciation, mineralogy, different ageing times and contamination sources (Oomen et al., 2002; Reeder et al., 2006; Tang et al., 2007b; Meunier et al., 2010).

Table 3.3 Relationships (goodness of fit, R^2) between Pb bioaccessibility and selected soil properties.

Soil property	Gastric bioaccessibility		Intestinal bioaccessibility		Intestinal bioaccessibility without WRA soil data	
	r^2	*p*	r^2	*p*	r^2	*p*
pH	0.09	0.21	0.00	0.56	0.08	0.02
Clay (%)	0.01	0.37	0.24	<0.0001	0.11	0.0044
Silt (%)	0.01	0.24	0.04	0.055	0.01	0.36
Sand (%)	0.01	0.27	0.10	0.0036	0.04	0.11
EC (us cm^{-1})	0.04	0.1	0.18	<0.0001	0.25	<0.0001
TC (%)	0.14	0.0028	0.38	<0.0001	0.48(-)	<0.0001
TOC (%)	0.24	<0.0001	0.19	<0.0001	0.45(-)	<0.0001
DOC (mg kg^{-1})	0.06	0.048	0.04	0.06	0.12	0.0035
CEC (cmol kg^{-1})	0.10	0.017	0.5447(-)	<0.0001	0.50(-)	<0.0001
Fe oxide (g kg^{-1})	0.35	<0.0001	0.11	0.0021	0.41(-)	<0.0001
Al oxide (g kg^{-1})	0.34	<0.0001	0.14	0.0004	0.43(-)	<0.0001
Mn oxide (g kg^{-1})	0.07	0.046	0.29	<0.0001	0.20	0.0001
Total Fe (g kg^{-1})	0.18	<0.0001	0.07	0.018	0.23	<0.0001
Total Al (g kg^{-1})	0.07	0.014	0.14	0.0004	0.16	0.0005
Total Mn (g kg^{-1})	0.09	0.0087	0.37	<0.0001	0.27	0.0001
Total P (g kg^{-1})	0.53	0.07	0.23	0.28	0.50	0.11

"-"means negative relationship. Highlighted values represent strong relationship (r^2 >0.5, p<0.0001)) between Pb bioaccessibility and soil property. "n" used in regression model is 7 (seven types of soils) for each pair of soil property and Pb bioaccessibility.

3.3.4 Effects of As and Cd on the bioaccessibility of Pb during UBM extraction

Results of interaction studies between As and Pb as well as between Cd and Pb are summarised in Table 3.4. Statistical comparison using t-test (Table S5 in Appendix 3) demonstrates that effect of As or Cd on the bioaccessibility of Pb was insignificant (p>0.05) in all types of soils. Vice versa, Pb did not have any impact on As or Cd bioaccessibility either (Table S4 in Appendix 3). Even though soils have varying properties, no interaction was observed in any type of soil, which indicates similar activity of As, Cd and Pb during bioaccessibility extraction regardless of soil type. It seems when soils were spiked with As or Pb to individual soils of the same type and aged separately, As and Pb would interact with ligands in soils independently. Therefore As and Pb was inclined to stay with their individual binding sites during UBM extraction when interaction study was performed by mixing up equal amounts of the same type of soil spiked with As or Pb in the same extraction container.

Lead arsenate ($PbHAsO_4$), which is insoluble in water, was unlikely to be formed in the intestinal phase of this *in vitro* bioaccessibility system. The possible explanation was that Pb maybe precipitate with inorganic salts (e.g. carbonate, sulphate) or combine with thiol groups of proteins (e.g. bovine serum albumin, bile) in UBM fluids (Belatik et al., 2012), thus significantly decreasing the concentration of free Pb^{2+} which can form precipitate with $HAsO_4^{2-}$. Oomen et al. found Pb in the artificial digestive fluid largely combined with bile (Oomen et al., 2003b). This outcome of null-interaction is not surprising considering Chapter 2 reported that there was no interaction between As and Cd in seven types of soils which underwent the independent ageing and extraction process as in this study probably because concentrations of free Cd^{2+} and AsO_4^3 were lower than the solubility (Ksp) of cadmium arsenate. For Pb and Cd, they are both divalent cations and competition for binding sites may occur to some extent during extraction. The reason why there was no observed interaction between Cd and Pb may be due to concentrations of binding sites being much higher than solubilised Cd (10^{-7} to10^{-6} mol L^{-1}) or Pb (10^{-7} to10^{-5} mol L^{-1}). Taken together with the results in Chapter 2, it suggests that under spiked concentrations in this study, additive effect can be assumed to calculate the bioaccessibility of any binary mixture of As, Cd and Pb when children ingest soils that contain independently-aged As, Cd and Pb, e.g. children ingest soil particles containing As at one site prior to ingestion of Pb-containing soil at another spot or soils containing different contaminants which enter the same soil at long time intervals. Although UBM highly simulates human digestive condition, it is an *in vitro* system and cannot represent the real environment in humans. Furthermore, the manner by which soils were spiked should be further studied. In this study, aliquots of the same soils were spiked with single As, Cd and Pb therefore As/Cd/Pb aged independently. However, contaminants may be released from the same source then they are aged in soil simultaneously. The interaction effects of As and Cd on the resultant bioaccessibility of Pb under this scenario remain to be investigated by sequentially spiking As, Cd and Pb into the same soil. Last but not the least, concentrations of mixed contaminants maybe of importance since competition for binding sites could take place at higher concentrations thereby change interaction results, which should be studied with priority in future work.

Table 3.4 Effects of As and Cd on the bioaccessibility of Pb during UBM extraction

	Pb(As) bioaccessibility			Pb(Cd) bioaccessibility		
Soil code	Total Pb (mg kg^{-1})[a]	Gastric bioaccessibility (%)[b]	Intestinal bioaccessibility (%)[b]	Total Pb (mg kg^{-1})[a]	Gastric bioaccessibility (%)[b]	Intestinal bioaccessibility (%)[b]
MIA	680	55±1.4	13±0.7	680	55±1.2	13±0.7
	680(69)	55±4.6	11±4.5	680 (51)	51±2.2	12±6.6
	5949	99±2.4	31±3.1	3367	86±1.8	16±1.6
	5949(732)	92±1.4	26±1.0	3367(332)	88±14.1	17±6.3
MGA	453	48±0.3	8±2.0	453	48±0.3	8±2.0
	453 (128)	45±1.5	7±2.7	453 (38)	36±6.1	8±1.1
	7028	65±6.3	9±3.9	7028	65±6.3	9±3.9
	7028 (313)	63±0.7	9±1.2	7028(833)	53±1.1	6±0.1
KBA	569	69±1.8	18±2.5	569	69±1.8	18±2.5
	569 (80)	70±3.3	14±1.8	569 (61)	62±6.8	16±1.9
	4544	91±9.3	24±3.6	4544	91±9.3	24±3.6
	4544(1051)	84±2.1	21±0.9	4544(671)	80±10.2	22±2.2
TAA	309	74±2.4	18±0.6	309	74±2.4	18±0.6
	309 (76）	80±4.0	18±5.7	309 (76）	65±3.2	17±2.8
	3997	74±2.6	16±3.2	3997	74±2.6	16±3.2
	3997 (903)	80±4.8	13±1.8	3997(775)	70±2.7	16±0.1
WRA	289	92±3.2	2±0.9	289	92±3.2	2±0.9
	289 (80)	92±3.2	2±1.3	289 (50)	86±0.7	3±2.3
	3898	99±10.3	2±0.2	3898	99±10.3	2±0.2
	3898 (522)	93±2.8	2±0.7	3898(570)	86±5.7	3±1.0
PBA	445	71±6.2	15±3.0	445	71±6.2	15±3.0
	445 (86)	70±3.6	12±1.0	445 (41)	64±5.8	14±0.8
	3971	90±7.1	18±0.4	3971	90±7.1	18±0.4
	3971 (247)	80±3.0	18±1.8	3971(338)	79±6.8	18±1.9
DUA	443	78±12.9	35±1.7	443	78±12.9	35±1.7
	443(101)	87±2.0	28±6.4	443 (75)	77±10.4	31±1.4
	4361	87±0.3	29±6.3	4361	93±7.0	29±6.3
	4361(248)	81±3.6	32±2.0	4361(413)	83±2.0	32±2.2

"Pb(As) bioaccessibility", 680(69) means 0.15 g soil spiked with 680 mg kg^{-1} Pb was mixed with 0.15 g soil spiked with 69 mg kg^{-1} As, etc; "Pb(Cd) bioaccessibility", 680(51) means 0.15 g soil spiked with 680 mg kg^{-1} Pb was mixed with 0.15 g soil spiked with 51 mg kg^{-1} Cd, etc.

[a] Data represent the mean of duplicate measurements.

[b] Data represent the mean of at least triplicate measurements ± standard deviation (SD).

The <250 μm particle size was used for all measurements.

3.4 Conclusion

Linear regression indicates that total content of Pb was the most important parameter for determining bioaccessible Pb. Soil properties such as organic carbon, Fe/Al oxides seemed to be negatively impact Pb bioaccessibility. Except for these soil properties, mineralogy (carbonate) was observed to play a more important role in Pb bioaccessibility. However, these conclusions were based on results of 7 types of soils and need be further strengthened by including more soil types in the future. When soil containing As or Cd co-existed with soil containing Pb during UBM extraction, Pb bioaccessibility was not affected by either As or Cd. Vice versa, neither As nor Cd bioaccessibility was influenced by the existence of Pb. This finding suggests when As, Cd or Pb were spiked in individual samples of the same soil type and aged independently, they would not interact with each other in the UBM simulated digestive system. Therefore, additive effect can be proposed when it comes to the bioaccessibility of these two binary mixtures which are aged independently and ingested simultaneously. However, this assumption needs to be applied with caution before animal validation studies are carried out. Future work is highly recommended with regard to spiking manners and higher spiked concentrations.

Chapter 4 Interaction effects of As, Cd and Pb on their respective bioaccessibility with time in co-contaminated soils assessed by the Unified BARGE Method

Under review by *Environmental Science and Pollution Research*

Abstract

Interaction effects of As, Cd and Pb on their respective bioaccessibility in co-contaminated soils were reported. In addition, the influence of ageing time (up to 90 days) on potential interactions was also investigated. Experiments were carried out by spiking four diverse soils with single, binary or ternary mixtures of As, Cd and Pb. Soils were measured for bioaccessibility at different ageing periods. Results demonstrate that bioaccessibility of As, Cd and Pb reached a relatively steady state after soils were aged for 90 days. Bioaccessibility of As, Cd and Pb in soils spiked with binary mixtures of As, Cd and Pb were not affected by the other co-existing metal/metalloid. But when As, Cd and Pb were introduced together to acidic soils which did not have abundant binding sites, intestinal bioaccessibility of Cd was increased temporarily at the early stage of ageing (7 to 30 days) whilst bioaccessibility of As and Pb remained unchanged. However, when Pb and As were added after Cd had been incubated in soil for 7 days, Cd intestinal bioaccessibility was not influenced by As and Pb. Therefore a number of factors should be taken into consideration when estimating the bioaccessibility of simultaneously-aged As, Cd and Pb, including the loadings of As, Cd and Pb in soils, the time length for which they have been aged together and the time period between As, Cd and Pb entering soils. In comparison with Chapters 2 and 3, this chapter provides further information on mixed contaminants (simultaneous ageing).

4.1 Introduction

During the last century, a great number of contaminants have been accumulated in soil due to industrial, agricultural and urban activities, such as manufacture, metal processing, mining, pesticide and landfill. It has been reported that over 150,000 sites are potentially contaminated in Australia (Carbonell, 2013). Furthermore, 60 to 80% of these sites are found in urban areas, which may specially put children at higher risks due to their hand-to-mouth activities (Calabrese et al., 1989; Rodriguez and Basta, 1999). Soils contaminated by As, Cd and Pb have received great attention because they are toxic to humans and often occur together at contaminated sites (ATSDR, 2004; Thavamani et al., 2011). Ingestion is regarded as the major exposure pathway for soil-bearing contaminants. When it comes to assessing the risk posed by soil contaminants via ingestion, there is a growing consensus that total concentrations of contaminants are of limited value. Therefore, estimating bioavailability, which represents the portion of contaminant that reaches the circulatory system, is essential in human health risk assessment. However, animal experiments measuring bioavailability require a significant input of time and budget and are becoming increasingly inaccessible due to ethical concerns. Since bioaccessibility (the soluble part of contaminant in human gastrointestinal tract) is the key limiting parameter that influences the bioavailability, *in vitro* bioaccessibility models have been developed to predict *in vivo* uptake after being correlated with *in vivo* bioavailability data (Ruby et al., 1996; Rodriguez and Basta, 1999; Juhasz et al., 2009; Denys et al., 2012).

Bioaccessibility of As, Cd and Pb in soils can be impacted by a number of factors (pH, organic matter, clay, oxides, ageing time, etc.) and these bioaccessibility-soil relationships have been previously investigated (Sarkar et al., 2007; Roussel et al., 2010; Das et al., 2013). However, very little attention has been paid to the effects of As, Cd and Pb on their respective bioaccessibility in co-contaminated soils, i.e. whether the existence of one metal/metalloid can influence the solubility of another co-existing metal/metalloid in gastrointestinal tract. Overlooking this interaction may result in an overestimation or underestimation of the risk posed by mixed contaminants. Furthermore, the reaction period should be considered since the longer they are incubated (co-exist) together, the higher possibility there is for variation in the interactions. Effects of ageing on the bioaccessibility of single As, Cd or Pb in soil have been studied (Tang et al., 2006a; Tang et al., 2007a; Tang et al., 2008) but it remains unclear whether the presence of other metal/metalloid could modify the temporal variation in the bioaccessibility of these single contaminants.

In this study, four variant types of Australian soils were spiked with single, binary or ternary mixtures of As, Cd and Pb. Soils were aged at ambient temperature and bioaccessibility was measured at different time points (up to 90 days) with the aim to investigate the effects of As, Cd and Pb on their respective bioaccessibility over time. In order to keep consistent with Chapters 2 and 3, bioaccessibility of As, Cd and Pb was also measured by UBM in this chapter. This study is a follow-up of Chapters 2 and 3 in which the interactions among binary mixtures of independently-aged As, Cd and Pb during the UBM extraction were investigated and would provide further invaluable information for risk assessment of mixed soil contaminants which are aged in a concurrent manner.

4.2 Materials and Methods

4.2.1 Soil sampling and characterisation

Background control soils were collected from four locations in Victoria and South Australia, namely Dublin (DUA), Kersbrook (KBA), Mount Gambier (MGA) and Tarrington (TAA). Details are available in Chapters 2 and 3, regarding soil sampling map, soil processing, storage, texture classification and characterisation of pH, total carbon (TC), total nitrogen (TN), total sulphur (TS), total organic carbon (TOC), dissolved organic carbon (DOC), cation exchange capacity (CEC), oxalate-extractable Fe/Al/Mn (representative of the amorphous Fe/Al/Mn oxide contents), total Fe/Al/Mn concentrations and particle size distribution.

4.2.2 Sequential spiking of As, Cd and Pb into soils

Arsenic (sodium arsenate, $Na_2HAsO_4 \cdot 7H_2O$), Cd (cadmium nitrate, $Cd(NO_3)_2 \cdot 4H_2O$) or Pb (lead nitrate, $Pb(NO_3)_2$) were purchased from Sigma-Aldrich, Australia. In each type of soil, As, Cd and Pb were spiked individually or sequentially as mixtures (Table 4.1). Take Cd + As for example, solutions of Cd were added to soils followed by continuous mixing using a plastic rod. Samples were kept in the dark for 24 hours (spike time interval) to allow Cd to move from solution phase to soil phase. Then soils were freeze-dried and solutions of As were introduced followed by continuous mixing. Afterwards, soils were aged at ambient temperature and maintained at water contents of approximately 60-70% (w/w) of field capacity by periodical replenishment of water. The spiked concentrations of As, Cd, Pb were 100, 150 and 1000 mg kg^{-1} respectively. The controls (non-spike) of each soil were prepared

and stored in the same manner. A different spike time interval, 7 days, was used for soil samples discussed in Section 4.3.4 in this chapter. The aim was to elucidate whether the interaction among As, Cd and Pb would be modified when the time interval between metal/metalloid addition was altered, reflecting time sequence of co-contamination which may occur in the real environment.

Previous studies indicate freeze-drying process may affect soil aggregate stability and microstructure of clays (Kang et al., 2003; Dagesse, 2011). But there is no literature demonstrating freeze-drying could influence bioaccessibility. In fact, existing data show that there is no significant difference in Pb bioaccessibility between air-dried and freeze-dried soils (Furman et al., 2007). In addition, due to small size of soil samples, the time for freeze-drying soils can be completed within several hours. Also, control samples have been prepared at the same time. Therefore, freeze-drying is not considered as an issue in this chapter.

Table 4.1 Sequence by which binary or ternary mixtures of As, Cd and Pb were added to soils 24 hours apart

Treatment	1st metal/metalloid added	2nd metal/metalloid added	3rd metal/metalloid added
Cd	Cd	—	—
As	As	—	—
Pb	Pb	—	—
Cd + As	Cd	As	—
Cd + Pb	Cd	Pb	—
Pb + As	Pb	As	—
Cd + Pb + As	Cd	Pb	As

4.2.3 Analysis of bioaccessibility with time

Aliquots of soils were sampled at the time points of 7, 30 and 90 days and sieved mechanically to obtain the <250 μm fraction for the analysis of total concentrations and bioaccessibility. The selection of 90 days, was based on the literature that reduction in bioaccessibility was largely completed by 3 months (Yang et al., 2002; Tang et al., 2008). UBM (Appendix 1) was initially designed for 0.6 g of each soil sample to be extracted in 60 mL UBM simulated solution. However in this study, the procedure was adapted for 0.3 g

subsample and 30 mL UBM solution. Both gastric and intestinal bioaccessibility were reported in this chapter.

4.2.4 Quality assurance (QA) and quality control (QC)

Please refer to Section 3.2.4 (page 62). Table S1 in Appendix 4 summarises the results of soil certified materials.

4.2.5 Statistical analysis

Please refer to Section 3.2.5 (page 62).

4.3 Results and discussion

4.3.1 Soil selection

A detailed description of soil locations, textures and physio-chemical properties can be found in Sections 2.3.1(pages 48-49) and 3.3.1(page 63). We selected four types of soils out of seven types of soils, namely MGA, KBA, TAA and DUA. Key soil characterisation of these four soils is summarised in Table 4.2. The reason why these four types of soils were chosen for this chapter was that they had distinct soil parameters in terms of total organic carbon (TOC), Fe oxide, Al oxide, pH which have been observed as important controllers for bioaccessibility of As, Cd and Pb reported in previous chapters. MGA soils contained a high amount of TOC (8.37 %), Fe oxide (12.49 g kg^{-1}), Al oxide (12.6 g kg^{-1}) whilst few TOC (1.48 %), Fe oxide (0.64 g kg^{-1}) and Al oxide (0.74 g kg^{-1}) were detected in DUA soils. KBA and TAA soils possessed a moderate amount of TOC, Fe oxide and Al oxide. KBA soils showed a higher value of TOC (5.5 %) than TAA soils (4.97 %) whilst TAA soils contained more Fe oxide (3.12 g kg^{-1}) and Al oxide (3.01 g kg^{-1}) than KBA (Fe oxide, 1.72 g kg^{-1}; Al oxide, 2.13 g kg^{-1}). DUA soils had a slightly alkaline pH while KBA, MGA and TAA soils were moderately acidic. According to the distribution of clay, silt and sand, these four soils were categorised into loam (KBA), loamy sand (DUA) and sandy loam (MGA and TAA). Total Mn and Mn oxide did not exist in a great amount in any of the studied soils whilst total contents of Fe and Al were in the range of 9.87 to 43.9 and 7.98 to 56.64 g kg^{-1}, respectively. There was no significant correlation between total Fe and Fe oxide as well as between total Al and Al oxide (p>0.05).

Table 4.2 Key soil parameters of four types of soils

Soil code	MGA	KBA	TAA	DUA
Soil location	Mount Gambier	Kersbrook	Tarrington	Dublin
pH	5.68	4.45	4.92	7
USDA texture*	Sandy loam	Loam	Sandy loam	loamy sand
TC (%)	8.37	5.5	4.97	2
TOC (%)	8.37	5.5	4.97	1
TIC (%)	0	0	0	0
Fe oxide (g kg^{-1})	12.49	1.72	3.12	1
Al oxide (g kg^{-1})	12.6	2.13	3.01	1
Mn oxide (g kg^{-1})	0.302	0.0449	0.212	0
Total Fe (g kg^{-1})	30.95	22.65	43.9	10
Total Al (g kg^{-1})	26.99	11.13	56.64	8
Total Mn (g kg^{-1})	0.5	0.06	0.27	0

* USDA, the United States Department of Agriculture soil classification; Data represent the mean of duplicate analysis. Values varied by less than 5%.

4.3.2 Temporal variations in bioaccessibility of As, Cd and Pb in their individually-spiked soils

The most significant reduction in bioaccessibility of As, Cd and Pb finished in the first 7 days of ageing. Bioaccessibility in soils reached a steady state within 30-90 days of ageing (Table 4.3). This was in accordance with previous researchers who have reported that bioaccessibility of As, Cd and Pb levelled off when soils were aged for 1 to 3 months (Yang et al., 2002; Tang et al., 2006c; Tang et al., 2007b; Tang et al., 2008). In addition, it is noted that the bioaccessibility of As, Cd and Pb measured in soils which were aged for 90 days were similar to those aged for one year using the same soils reported in Chapters 2 and 3. The uniformity in bioaccessibility between 90 days and one year indicates no pronounced metal/metalloid-soil interaction that may lead to a significant decrease in bioaccessibility after 90 days in this current study. Also, in the previous chapters, it is demonstrated that TOC, Fe oxide and Al oxide can decrease the bioaccessibility of As, Cd and Pb as measured by UBM. These relationships were also observed here with MGA soils which had the highest amount of TOC, Fe oxide and Al oxide among the four types of soils showing the lowest bioaccessibility of As, Cd and Pb. Moreover, within the four types of soils in this study, Fe

and Al oxides seemed to play a more important role than TOC. Bioaccessibility of As, Cd and Pb in TAA soils was found to be lower than those in KBA soils. When comparing their soil properties, it is worthy of notice that TAA soils possessed higher contents of Fe and Al oxides but lower organic carbon than KBA soils. Arsenic, Cd and Pb in DUA soils were most bioaccessible since it contained apparently fewer binding sites (i.e. lower organic carbon, Fe and Al oxides). Gastric bioaccessibility of Cd and Pb was generally observed to be much higher than those of As because, under acidic gastric condition (pH = 1.2-1.5), the soil surfaces would be more positively charged than at ambient soil pH, which may inhibit Cd and Pb but enhance As sorption on soil. Furthermore, Cd and Pb were under direct competition with H^+ at solid-solution interface and the high H^+ activity under gastric conditions would displace Cd and Pb. By contrast, As binds to mineral surfaces via ligand exchange and the dissolution of mineral surfaces is likely to be a more significant process in gastro-induced desorption. Dissolution of As from oxide surfaces would be incomplete (low As gastric bioaccessibility) within the time frame of acid extraction (Cornell and Schwertmann, 2003) whilst significant metal-H^+ replacement would occur rapidly (high Cd and Pb gastric bioaccessibility). When comparing bioaccessibility of Cd and Pb, bioaccessibility of Pb in gastric phase was lower than that of Cd. Especially in MGA soils, nearly half of the Pb was not bioaccessible while Cd remained highly soluble. Previous study indicates that Pb was retained in soils stronger than Cd which may be attributed to its greater hydrolysis constant, higher atomic weight and ionic radius, and larger Misono softness value over Cd (Shaheen, 2009). These parameters favoured Pb to more readily undergo inner-sphere surface sorption and complexation than Cd.

Table 4.3 Temporal change in bioaccessibility of single As, Cd and Pb with ageing time

Soil code	Ageing time (d)	As bioaccessibility (%)		Cd bioaccessibility (%)		Pb bioaccessibility (%)	
		Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase
MGA	7	43±0.8	21±3.2	93±2.2	7.1±1.1	50±2.2	8.5±1.2
	30	41±1.9	17±2.3*	95±0.4	7.0±2.1	51±1.2	8.2±0.1
	90	42±2.1	17±1.2	96±3.0	9±0.1	48±0.9	7.4±0.1
KBA	7	87±1.7	83±4.2	83±1.8	22±1.9	80±1.5	22±0.7
	30	62±8.3**	68±2.0**	84±1.3	18±0.1*	77±1.9	19±1.1
	90	67±0.7	54±6.5*	85±1.1	25±1.0**	78±3.3	20±2.6
TAA	7	60±1.0	48±5.3	92±1.4	16±2.8	79±2.3	19±1.9
	30	48±0.2**	43±1.0	84±3.1*	18±1.5	77±2.0	18±0.7
	90	44±4.4	43±0.1	88±5.9	18±2.1	82±0.5	17±1.7
DUA	7	92±1.1	83±0.4	93±0.8	34±4.8	80±0.4	29±2.9
	30	86±1.6*	80±0.8	93±6.0	37±4.2	82±4.3	32±0.2
	90	84±0.1	79±10	90±2.4	37±1..3	78±0.1	30±2.3

Data represent the mean of triplicate measurements ± standard deviation (SD). Data with asterisk (*) mean that bioaccessibility at this time point was significantly different from that measured at previous time point (**, $p<0.005$; *. $p<0.05$).

4.3.3 Interaction effects of As, Cd and Pb on their respective bioaccessibility in co-contaminated soils

Tables 4.4-4.6 summarise bioaccessibility of As, Cd and Pb in their co-spiked soils aged up to 90 days. Gastric bioaccessibility of As, Cd and Pb in all co-contaminated soils was not significantly different from that in soils spiked with individual As, Cd or Pb at corresponding ageing time ($p>0.05$). When soils progressed from the gastric phase to intestinal phase, bioaccessibility of As, Cd and Pb in soils spiked with binary mixtures of As, Cd and Pb was not affected by each other. For example, intestinal bioaccessibility of Cd in KBA soil measured at 7 days (Table 4.3) was 22±1.9 %, which did not differ significantly ($p>0.05$) from that (22±1.7 %) in soil spiked with both Cd and As (Table 4.5). Liang et al. (2014) found that when As (40 mg kg^{-1}) and Pb (150 mg kg^{-1}) were spiked together in soils which contained 0.1-1.2 g kg^{-1} Fe oxide and 0.32–1.3 % organic carbon, the distribution of As in soil fractions (non-specifically sorbed As, specifically sorbed As, amorphous and poorly-crystallized Fe/Al-bounded As, well-crystallized Fe/Al-bound As and residual As) was

similar to that of single As (40 mg kg^{-1}) and Pb distribution (exchangeable Pb, carbonate-bound Pb, Fe/Mn hydroxide-bound Pb, organic-bound Pb and residual Pb) was paralleled to that of single Pb (150 mg kg^{-1}). In our study, soils contained 0.64-11.49 g kg^{-1} Fe oxide and 1.48-8.37 % organic carbon which were significantly higher than the soils used by Liang et al. Therefore, a possible explanation for the null interaction could be that As, Cd and Pb partitioned into different soil fractions independently when there were plenty of reaction sites for As, Cd and Pb loaded in soils. Also, since As, Cd and Pb solutions were added into soils sequentially with 24 hours (spike time interval) apart rather than concurrently in one mixed-solution, insoluble compounds such as Cd/Pb arsenate were less likely to be formed in soils due to the rapid sorption of metals/metalloid from solution to soil phase (Bradl, 2004).

However, when Pb and As were added to Cd-spiked soil one after another, intestinal bioaccessibility of Cd in KBA and TAA soils increased relative to soils spiked with single Cd at the early stage of ageing (data in Table 4.5 marked with *). In contrast, bioaccessibility of As and Pb was not influenced in the presence of Cd. Compared to binary mixtures of As, Cd and Pb, the co-existence of As, Cd and Pb in the same soil sample may lead to competitive sorption on soils due to the increased loading of metals/metalloid. Prior studies have noted that Pb was absorbed in preference to Cd thus increasing the observed solubility of Cd (Serrano et al., 2005; Appel et al., 2008). In this study, this preference was reflected in the observation that bioaccessibility of Pb in four types of soils was stable after 7 days ageing, which indicates a fast reaction between Pb and soil components. Besides, strong affinity of As for organic carbon, Fe oxide and Al oxide has been extensively reported (Solaiman et al., 2009; Komarek et al., 2013). Therefore, there was a high possibility that when they were introduced simultaneously to soil spiked with Cd, As and Pb may interfere Cd sorption to soil functional groups, resulting in unbound or less strongly adsorbed (e.g. more labile) Cd that was more bioaccessible during the UBM extraction. Another possible reason could be that soils spiked with ternary mixture of As, Cd and Pb were better homogenised (one more time continuous mixing using a plastic rod when adding the third contaminant) than soils spiked with binary mixture of As, Cd and Pb, which increased the possibility that As, Cd and Pb occurred in the same space of soils. However as ageing continued, bioaccessibility of As and Pb reached a steady stage where As and Pb finished interacting with soil binding sites, thus gave way to Cd sorption. Therefore, at the end of 90 days, the bioaccessibility of Cd in soils spiked with As, Cd and Pb was the same as that in soils spiked with single Cd. This implies

that the time length for which As, Cd and Pb co-existed is an important factor to be considered in addition to loadings of As, Cd and Pb.

The aforementioned phenomenon was not observed in another acid soils, MGA, which may be attributed to the significant amounts of organic carbon, Fe oxide and Al oxide in this type of soil. Interestingly, even though DUA soils contained few sorption sites than the other three types of soils, it seems interaction did not take place among As, Cd and Pb during ageing. It is postulated that under the alkaline condition of DUA soils (pH=7.31), high pH may favour Cd to interact with soil components instantly, thereby leaving less chance to be affected by subsequent As and Pb (Bradl, 2004). On the contrary, acidic soil (KBA and TAA) surface was more positively charged, so it might keep Cd from developing strong complex with soil surface functional groups during early ageing period, thus Cd was vulnerable to the effects of As and Pb (Lu et al., 2005; Tang et al., 2006c).

Taken together, when loadings of As, Cd and Pb reached certain concentrations, As and Pb (mostly Pb) would interfere with the sorption of Cd in soils at early stages of ageing in acidic soils, leading to elevated Cd intestinal bioaccessibility. But this interaction was not pronounced after soils were continued to be aged for a longer time, 90 days in this study. That is to say, the time for Cd to reach a steady intestinal bioaccessibility was prolonged in the presence of As and Pb. However, soils containing a large amount of organic carbon, Fe oxide and Al oxide or alkaline pH seemed to be free of the above-mentioned interaction. These results contrasted with what was observed in Chapters 2 and 3 in which soils were spiked and aged in an independent manner. Take Chapter 2 for example, As and Cd was spiked individually in separate aliquots of the same type of soils. Equal portions of As-spiked and Cd-spiked soils were weighed and mixed together in the same container then extracted using UBM. Under this scenario, As and Cd aged independently and only co-existed in the simulated human digestive fluids. Results show neither As nor Cd bioaccessibility was impacted by the existence of the other one at varying concentrations. Combining findings of previous and current chapters, it is therefore we could assume additive effect among mixed contaminants of As, Cd and Pb for health risk assessment purposes under the following five situations: (1) As, Cd and Pb are aged independently, e.g. there is a long time gap between As/Cd/Pb entering the same soil or they are aged at different soils; (2) As, Cd and Pb are aged concurrently but loadings do not exceed the sorption capacity of soils; (3) As, Cd and Pb are aged together in soils which are alkaline; (4) As, Cd and Pb enter the same soil at long

(>7 days) time intervals and aged simultaneously; (5) As, Cd and Pb are simultaneously aged for a long time (90 days in this chapter).

Key questions which need further investigation, but are out of the scope of the present study include: (1) whether the order by which As, Cd and Pb are introduced into soils can influence the interaction pattern; (2) whether mixtures are added into soil as one solution or separate solutions would make a difference; (3) spectroscopic evidence (e.g. synchrotron-based X-ray absorption spectroscopy) will help to understand the underlying interaction mechanism and speciation of As, Cd and Pb in soils; and (4) Validation is needed using *in vivo* bioavailability models.

Table 4.4 Temporal change in bioaccessibility of As with ageing time when soils were spiked with binary or ternary mixtures of As, Cd and Pb

Soil code	Ageing time (d)	As (Cd) bioaccessibility (%)		As (Pb) bioaccessibility (%)		As (Cd+Pb) bioaccessibility (%)	
		Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase
MGA	7	44±1.1	23±3.0	42±2.6	24±1.2	41±3.8	24±1.6
	30	40±0.8	18±1.3	40±3.4	18±0.8	41±0.4	19±2.2
	90	40±0.3	19±2.5	40±0.2	17±0.7	43±0.4	17±1.7
KBA	7	86±3.0	82±0.3	93±0.6	84±2.0	89±2.8	79±0.9
	30	65±1.4	69±0.7	68±2.5	66±5.9	64±8.3	72±4.3
	90	63±6.1	58±0.8	66±4.0	52±2.4	66±0.5	55±0.9
TAA	7	62±1.1	46±5.5	62±1.0	55±1.4	65±2.3	58±0.2
	30	45±1.1	43±1.3	48±0.8	45±1.8	48±2.9	46±0.5
	90	42±2.3	45±1.5	44±1.9	45±1.8	45±0.8	45±1.5
DUA	7	95±4.9	83±3.1	88±0.3	76±8.7	91±5.6	84±0.7
	30	93±6.0	80±1.3	90±3.2	81±1.6	92±1.5	82±0.1
	90	84±3.5	71±5.2	90±0.4	77±5.6	85±0.5	77±1.0

Data represent the mean of triplicate measurements ± standard deviation (SD). "As (Cd)" means soils were spiked with mixture of As and Cd; "As (Pb)" means soils were spiked with mixture of As and Pb; "As (Cd+Pb)" means soils were spiked with mixture of As, Cd and Pb. Bioaccessibility of As in mixtures was not significantly different from that of single As at corresponding ageing time (p>0.05).

Table 4.5 Temporal change in bioaccessibility of Cd with ageing time when soils were spiked with binary or ternary mixtures of As, Cd and Pb

Soil code	Ageing time (d)	Cd (As) bioaccessibility (%)		Cd (Pb) bioaccessibility (%)		Cd (Pb+As) bioaccessibility (%)	
		Gastric phase	Gastric+ intestinal phase	Gastric phase	Gastric+ intestinal phase	Gastric phase	Gastric+ intestinal phase
MGA	7	87±1.0	7.9±1.3	92±1.8	6.7±1.3	93±4.3	8.1±0.5
	30	85±2.9	6.8±0.3	92±6.1	7.4±1.0	86±4.2	8.1±0.4
	90	91±0.6	10±2.0	93±1.6	10±0.1	91±1.9	10±0.2
KBA	7	86±5.5	22±1.7	87±1.6	23±2.2	89±0.7	27±1.4**
	30	86±0.1	19±0.8	83±0.4	20±2.3	88±0.7	26±1.7**
	90	85±1.5	25±0.4	86±0.3	25±0.4	88±0.7	24±1.5
TAA	7	90±1.5	16±1.6	88±1.2	18±0.7	91±0.2	23±1.0**
	30	89±0.1	18±1.3	87±10.6	18±0.9	85±3.2	19±0.4
	90	88±0.2	19±0.9	92±1.4	18±0.5	91±1.4	20±0.4
DUA	7	92±6.0	35±1.1	88±4.7	35±1.7	92±3.3	34±5.3
	30	93±6.4	39±0.6	86±7.0	39±1.1	88±1.6	35±1.3
	90	86±2.9	35±1.7	90±2.7	37±2.2	86±4.1	36±3.3

Data represent the mean of triplicate measurements ± standard deviation (SD). "Cd (As)" means soils were spiked with mixture of Cd and As. "Cd (Pb)" means soils were spiked with mixture of Cd and Pb. "Cd (Pb+As)" means soils were spiked with mixture of Cd, As and Pb. Data with asterisk (**) mean bioaccessibility of Cd in mixtures was significantly different from that of single Cd at corresponding ageing time (p<0.005); Data without asterisk mean bioaccessibility of Cd in mixtures was not significantly different from that of single Cd (p>0.05).

Table 4.6 Temporal change in bioaccessibility of Pb with ageing time when soils were spiked with binary or ternary mixtures of As, Cd and Pb.

Soil code	Ageing time (d)	Pb (Cd) bioaccessibility (%)		Pb (As) bioaccessibility (%)		Pb (Cd+As) bioaccessibility (%)	
		Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase
MGA	7	54±1.0	7.4±1.0	53±2.2	8.0±0.9	54±1.1	8.9±0.8
	30	49±0.8	7.1±1.4	47±3.5	8.4±0.1	51±2.0	8.8±0.3
	90	47±0.2	7.6±0.7	49±0.4	7.9±0.5	47±2.7	7.2±1.3
KBA	7	76±1.1	19±1.8	81±1.1	20±0.8	81±3.7	20±2.3
	30	75±0.7	20±0.9	80±1.8	18±0.4	79±1.4	18±1.2
	90	77±0.4	18±0.3	81±2.5	17±1.5	78±0.2	18±1.0
TAA	7	80±1.3	17±2.0	80±0.2	17±0.9	81±0.4	18±2.2
	30	77±3.3	17±1.3	73±1.6	16±1.9	76±0.9	16±2.2
	90	83±1.4	17±0.5	80±0.4	17±0.3	82±0.1	16±2.3
DUA	7	83±3.4	31±3.5	80±0.1	30±1.4	86±3.7	32±2.5
	30	80±5.8	33±1.6	83±4.3	33±0.3	83±1.4	32±0.5
	90	80±1.8	30±0.7	79±0.1	32±1.0	76±0.1	33±2.2

Data represent the mean of triplicate measurements ± standard deviation (SD). "Pb (Cd)" means soils were spiked with mixture of Pb and Cd; "Pb (As)" means soils were spiked with mixture of Pb and As; "Pb (Cd+As)" means soils were spiked with mixture of Pb, Cd and As. Bioaccessibility of Pb in mixtures was not significantly different from that of single Pb at corresponding ageing time (p>0.05).

4.3.4 The effect of spike time interval on the interaction among As, Cd and Pb in KBA and TAA soils.

As discussed in Section 4.3.3, interaction effects of As and Pb on Cd intestinal bioaccessibility were observed in KBA and TAA soils at ageing time of 7 to 30 days. We took a further step to investigate whether the time interval could affect this interaction. Therefore, As and Pb was introduced to soils after Cd had been incubated in soils for 7 days and bioaccessibility of Cd was measured after soils were aged with ternary mixture of As, Cd and Pb for another 7 days. Results show no appreciable effects of As and Pb on intestinal bioaccessibility of Cd were observed in KBA and TAA soils (Table 4.7). Since the most significant reduction in Cd bioaccessibility was observed within the first week in this study, 7 days maybe sufficient for Cd to enter micro and meso pores and establish stable surface species, thus reducing the chance to be interfered by incoming As and Pb. Therefore when As and Pb were introduced to soils 7 days later than Cd, they did not affect Cd partitioning in

soils. From what has been discussed above, it can be maintained that not only the loadings and the time length As, Cd and Pb co-existed but also the time interval of spiking are important for potential interaction. Whether interaction could take place depends on the time point when As and/or Pb enters into the Cd-spiked soil, i.e. it is before or after Cd establishes strong bindings with soil functional groups.

Table 4.7 The effect of spike time interval (7 days) on the bioaccessibility of Cd in KBA and TAA soils

Soil code	Cd bioaccessibility (%)		Cd (Pb+As) bioaccessibility (%)	
	Gastric phase	Gastric + intestinal phase	Gastric phase	Gastric + intestinal phase
KBA	89±0.1	27±0.2	89±0.4	27±0.7
TAA	95±3.3	23±0.2	91±0.6	21±1.1

"Cd" means soils were spiked with single of Cd. "Cd (Pb+As)" means soils were spiked with mixture of Cd, As and Pb. In soils spiked with mixtures, Cd had been aged in soils for 7 days before the introduction of Pb and As. After the entrance of Pb and As, soil was aged for another 7 days. No statistical difference (p>0.05) was detected between Cd bioaccessibility and corresponding Cd bioaccessibility in mixture of As, Cd and Pb.

4.4 Conclusion

The reduction in the bioaccessibility of As, Cd and Pb in spiked-soil was completed within 90 days of ageing from the initial spiking. Total organic carbon, Fe oxide and Al oxide were negatively related with the intestinal bioaccessibility of As, Cd and Pb. When As, Cd and Pb were spiked into soils sequentially at a time interval of 24 hours, no interactions on their respective bioaccessibility were observed in soils spiked with binary mixtures of As, Cd and Pb. However, when As and Pb was introduced together to the same soil where Cd has been added, inhibition in Cd sorption to soils would take place in acidic soils containing limited binding components (e.g. organic carbon, Fe oxide, Al oxide) before 30 days of ageing, leading to a temporary increase in Cd intestinal bioaccessibility. Contrastly, As and Pb bioaccessibility remained unaffected. The time interval between As, Cd and Pb entering the soils was also of great importance in determining the kind of interactions. It seems when As and Pb were introduced to soils 7 days after Cd, no effect on Cd bioaccessibility was

detected. In summary, key factors need to be considered when assessing the bioaccessibility of mixtures of As, Cd and Pb in soils, including the amounts of active binding sites, soil pH, loading concentrations, length of time that metals/metalloid co-exist and time intervals. These results provide further insight for risk assessment of mixed metals/metalloid but should be further validated due to the inherent limitation of the UBM being a simulated method. Moreover, the order by which metals/metalloid are spiked and increased loading of metals/metalloid are worthwhile to be investigated in the future.

Chapter 5 Uptake of UBM-extracted arsenic, cadmium and lead in HepG2 cells and their interactions during uptake

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Abstract

Soil contaminants such as arsenic (As), cadmium (Cd) and lead (Pb), pose a great threat to human health, especially to children. After these contaminants become bioaccessible, liver is the key organ for further metabolism. This chapter is a continuation of bioaccessibility studies conducted in previous chapters, which aims to explore uptake of As, Cd and Pb into target organ cell (liver) as well as the interaction effects of As, Cd and Pb on their respective uptake after they are solubilised in digestive system. In this study, the HepG2 cell line (human carcinoma liver cell) was selected as the *in vitro* representative of liver. Results show that the uptake of As and Cd followed a dose-dependent manner. The uptake of Cd ranged from 1.84±0.8 to 10.9±2.0 ng/10^5 cells which was significantly higher than that of As (0.14 ±0.017 to 2.07±0.50 ng/10^5 cells). Interaction study indicates the accumulation of Cd in HepG2 cells was decreased by As or Pb whilst uptake of As was not affected by either Cd or Pb. Results of Pb uptake were of limited value due to most of Pb precipitating in dosing solutions, which suggests speciation should be taken into consideration in *in vitro* system. Nonetheless, HepGe2 cell line could be a useful model to predict accumulation and interaction of soil-born As and Cd at the hepatic level.

5.1 Introduction

When soils containing As, Cd or Pb are ingested simultaneously into human digestive system, As, Cd and Pb show no effects on respective solubility either in the gastric or

intestinal phases, i.e. bioaccessibility, as observed in Chapters 2 and 3. However, it is far from clear whether interaction would take place among As, Cd and Pb during their accumulation in target organ. Liver is well-known for its metabolic and detoxification functions and has been reported as the important organ for distribution and metabolism of As, Cd and Pb (Underhill, 1914; Chen et al., 2005; Arroyo et al., 2012). It is assumed that contaminants in the liver can reflect their overall systemic levels (Grøn, 2003) therefore liver is the key organ that can be considered when investigating the accumulation of As, Cd and Pb in human body. Previous *in vivo* experiments using rat suggest additive or antagonistic effects between binary mixtures of As, Cd and Pb (Fairhall and Miller, 1941; Mahaffey et al., 1981; Elsenhans et al., 1987; Yanez et al., 1991). However, due to time and cost constraint, ethical concerns, species difference, increasing numbers of chemicals and mixtures of chemicals, *in vitro* liver models have been established to mimic *in vivo* response, including liver slices, immortalised cell lines, primary hepatocytes, three-dimension cell culture system, bioartificial livers, and co-culture systems (Soldatow et al., 2013). Immortalised cell lines and primary hepatocytes are the most widely-adopted *in vitro* models. HepG2 cell line is one of the immortalised cells which have been well-characterised and is commonly used as a model system. HepG2 cells are able to activate and detoxify xenobiotics and thus reflect the metabolism of xenobiotics in the human body better than other metabolically incompetent cells utilised in conventional *in vitro* assays (Dehn et al., 2004; Mersch-Sundermann et al., 2004; Baderna et al., 2011; Baderna et al., 2013). Therefore, HepG2 cells were selected as the representative of liver in this study to investigate the subsequent uptake of As, Cd and Pb on hepatic level as well as their possible interaction effects on their respective uptake. Results can help to indicate the effectiveness of HepG2 cell line in simulating *in vivo* response by comparing with data obtained from previous *in vivo* interaction experiments.

Throughout the literature, HepG2 cells were treated with chemicals which are solubilised in pure solutions (e.g. water, DMSO) and then added into cell culture medium. However, this treatment regime does not always reflect the real exposure situation. For example, soil contaminants may undergo a series of reactions (e.g. dissolution, absorption) in human digestive system and end up with several species. Therefore, in this study, HepG2 cells were exposed to As, Cd or Pb extracted by simulated digestive system (UBM) in order to understand the discrepancy in the uptake between As/Cd/Pb in pure solution and in digestive fluids.

To sum up, soils containing As, Cd or Pb was first extracted in simulated digestive fluids as described in Chapters 2 and 3. During the extraction, As, Cd and Pb dissolved from soils thereby became soluble in the digestive fluids, which were called UBM-extracted As, Cd and Pb. HepG2 cells were then exposed to this UBM-extracted As, Cd and Pb mixing with cell culture medium to explore the post uptake and interaction effects of As, Cd and Pb after human digestive system. To the author's knowledge, this is the first study that uses HepG2 cell line to investigate accumulation and interaction of As, Cd and Pb at hepatic level. This study would provide invaluable information for *in vitro* research area regarding the potential of HepG2 cell to mimic *in vivo* response. Furthermore, as the first study using digestive fluids for hepatocyte exposure, results can provide important implication by comparing with traditional *in vitro* studies using pure solutions.

5.2 Materials and Methods

5.2.1 Cell culture

HepG2 (HB-8065, ATCC, U.S.) cells were grown on 75 cm^2 culture flask and maintained at 37 ℃ in the incubator (Model HERAcell 150, Thermo Scientific, U.S.). The atmosphere in the incubator contained 5% CO_2. The cell culture was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Australia), amended with 10% fetal bovine serum (Gibco, Australia), 1% penicillin-streptomycin (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl). Passage was performed at the confluency of 70-80%.

5.2.2 Measurement of the solubility of UBM-extracted As, Cd and Pb in DMEM

Seven types of soils were extracted by UBM, namely Dublin (DUA), Kersbrook (KBA), Millicent (MIA), Mount Gambier (MGA), Port Broughton (PBA), Tarrington (TAA) and Wallaroo (WRA). Solutions obtained after incubation in the gastric phase of UBM as reported in Chapters 2 and 3 were utilised in this chapter. This represented the worst-case scenario as the gastric phase yields the highest solubilised concentrations of the target compounds. These solutions containing single or binary mixtures of As, Cd and Pb dissolved from soils were called UBM extraction solutions. UBM extraction solution was diluted with DMEM (v:v 1:9) without significantly changing the pH of DMEM thus affecting cell viability. The mixed solution of UBM extraction solution and DMEM (v:v 1:9) was referred

to as UBM-DMEM solution in this chapter. Each UBM-DMEM solution was divided into five aliquots. These aliquots were kept in the cell culture incubator then filtered through 0.45 μm filter (PVDF, Millipore, Australia) at 0.5, 6, 12, 24 and 48 hours. Samples were diluted using 2% HNO_3 and measured on ICP-MS. Arsenic (sodium arsenate), Cd (cadmium nitrate) and Pb (lead nitrate) in water solutions, which are referred to as pure As, Cd and Pb solutions in this study, was also added to DMEM for solubility study.

5.2.3 Uptake of As, Cd and Pb in HepG2 cells

Cells were seeded in 6-well plates at a density of 5×10^5 with DMEM (10% fetal bovine serum, 1% penicillin-streptomycin) for 24 hours. On the same day when cells were seeded, UBM-DMEM solutions were prepared and kept in the incubator for 24 hours. Then UBM-DMEM solutions were passed through 0.45 μm filter (PVDF, Millipore, Australia) and the filtrate was used to replace cell culture medium in the plate. After 24 hour exposure to UBM-DMEM solution, cells were washed with PBS containing 2 mmol L^{-1} EDTA, harvested by trypsin and then resuspended in fresh culture medium. An aliquot of 10 μL cell suspension was loaded on the dual-chamber counting slide (Bio-Rad, U.S.) and cell number was counted using a TC20 cell counter (Bio-Rad, U.S.). Cell suspension was then centrifuged at 130 g (Model 5810R, Eppendorf, Australia) for 5 minutes and the obtained cell pellet was resuspended in 2% HNO_3 for 24 hours to completely lyse the cells. Cell residues were removed by centrifugation and the supernatant was stored under 4 ℃ before ICP-MS analysis. Uptake of As, Cd and Pb in pure solutions was also measured. Results of uptake were normalised to per $1x10^5$ cells.

5.2.4 Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 (La Jolla, USA). Non-linear and linear regressions were carried out to explore the relationships between exposure concentrations and intracellular uptake. The 0.05 level of probability was used as a minimum criterion of significance.

5.3 Results and discussion

5.3.1 Solubility of UBM-extracted As, Cd and Pb in DMEM

Figure 5.1 shows the solubility of As, Cd and Pb in UBM-DMEM solution over time. The concentrations of As extracted from seven types of soils by UBM stayed at the initial concentrations in UBM-DMEM solution from 0 to 48 hours, which indicates As dissolved in digestive fluid may not interact with components in DMEM to produce precipitate. Possible explanation could be that As existed in forms of anion ($HAsO_4^{2-}$, AsO_4^{3-}) which were free from the effects of carbonate, sulphate, phosphate, etc. Moreover, measured concentrations of As in UBM-DMEM solution were in the magnitude of 10^{-6} mol L^{-1} which was lower than the solubility of some metal arsenate (e.g. Ksp[$Ca_3(AsO_4)_2$]= 0.003629, 25 ℃).

Unlike As, the soluble Cd in UBM-DMEM solution decreased significantly for the first 0.5 hour and gradually levelled off over the next 24 hours. The significant drop in the concentration of Cd was probably due to Cd forming precipitate with carbonate or phosphate. Also, Cd may also undergo binding reaction with thiol groups of protein or amino acids (e.g. bovine serum albumin, mucin, pepsin or their breakdown products) in UBM-DMEM solution. Once these reactions reached a balance, concentrations of Cd remained constant over time.

For Pb, 73-87% Pb extracted from soil by UBM precipitated out within 0.5 hour when it was added to DMEM. This reduction in Pb concentration kept on-going even after 24 hours. Inorganic ions in DMEM, such as phosphate, sulphate, carbonate which were in hundreds or thousands of mmol L^{-1}, may react with Pb to generate insoluble compounds during incubation time. Furthermore, CO_2 in the incubator may continually dissolve in the medium, thereby decreasing soluble Pb by forming lead carbonate, which may be the reason why Pb precipitate was continually generated over 48 hours.

The behaviour of pure As was the same as UBM-extracted As, which showed no change in its concentration for up to 48 hours (Figure 5.1). However, different from UBM-extracted Cd forming insoluble compounds, significant decrease in Cd concentration was not observed for pure Cd in DMEM, which was probably due to low levels of inorganic ions. Similar to UBM-extracted Pb, over 95% pure Pb became insoluble in the first 0.5 hour.

Taken the solubility issue of Cd and Pb into consideration, UBM-extracted As, Cd and Pb was added to DMEM for 24 hours and filtered through 0.45 μm filter in order to obtain

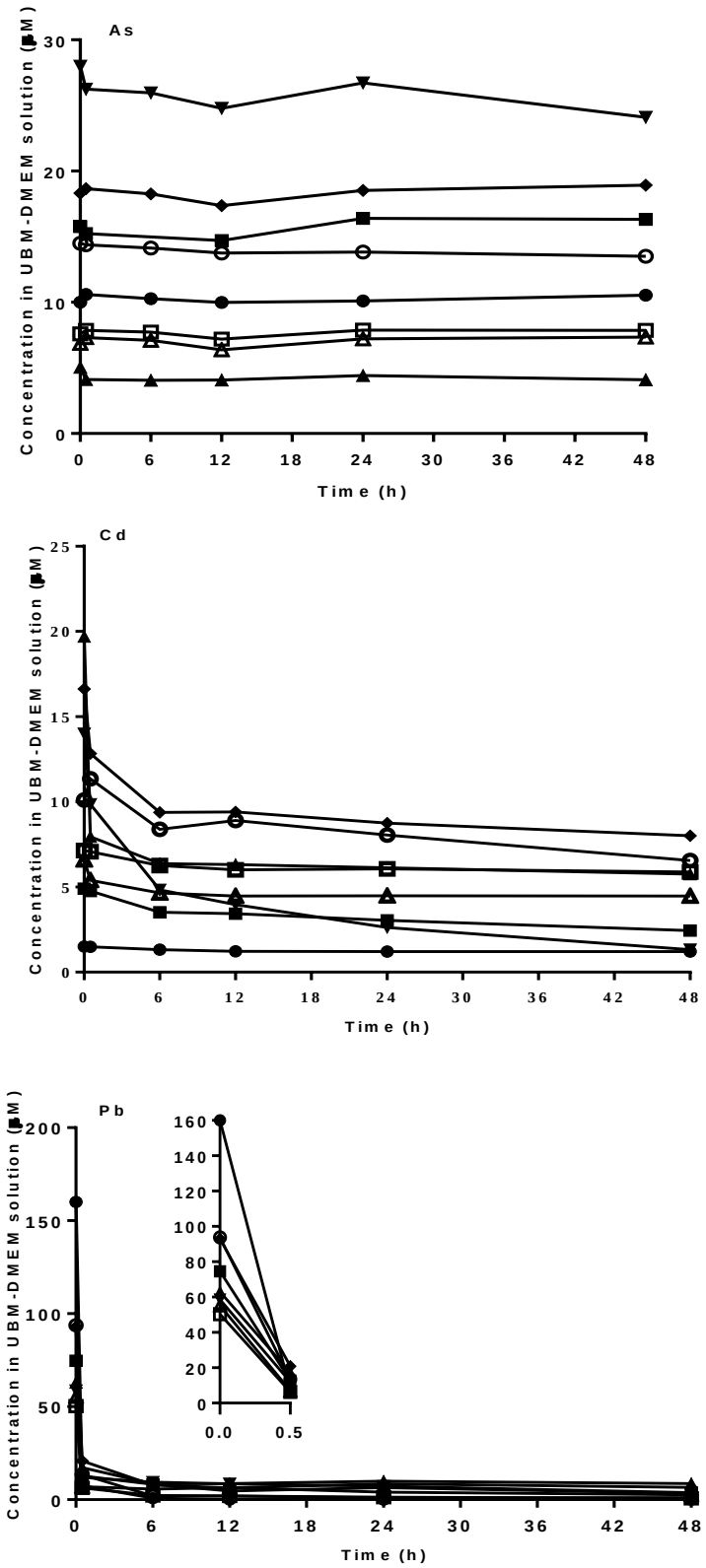

Figure 5.1 Temporal change in the concentrations of UBM-extracted As, Cd and Pb in DMEM. '●', '■', '▲', '▼', '◆', '○', '□', and '△' represent As/Cd/Pb in pure solution, or extracted from MIA, MGA, KBA, TAA, WRA, PBA and DUA, respectively. Each data represents mean of duplicate measurements.

stable dosing solutions. The solubility of UBM-extracted Cd in DMEM after 24 hours can be predicted from concentration of Cd in UBM solution (Figure 5.2). UBM-extracted Cd from MGA and WRA soils developed more precipitate in DMEM than those extracted from other soils. This may be attributed to the large amount of calcium (Ca), magnesium (Mg), iron (Fe) and aluminium (Al) in MGA soils (Table S1 in Appendix 5). These metals can form less soluble compounds with inorganic anions (e.g. CO_3^{2-}, PO_4^{3-}). The reduction in concentrations of these anions may enhance the dissociation of HCO_3^- and $H_2PO_4^-$ in the UBM-DMEM solution to generate more CO_3^{2-} and PO_4^{3-}. WRA soils were sampled from an area well known to have calcareous materials according to our collaborator. Calcareous soils are largely or partly composed of calcium carbonate. It was found that total Ca in WRA soils was the highest (49.48 g kg^{-1}) among the seven types of soils (Table S1 in Appendix 5). Calcium in UBM solution may react with CO_3^{2-} in DMEM thereby accelerating the dissociation of HCO_3^- to produce CO_3^{2-}. The produced CO_3^{2-} or PO_4^{3-} could precipitate with Cd and reduce the solubility of Cd. Contrast to MGA and WRA soils, PBA and DUA soils possessed few metals (i.e. Ca, Mg, Fe, Al), therefore the promotion of acid dissociation due to the formation of insoluble compounds was relatively insignificant. This could also explain the stability of pure Cd in DMEM since there were no other free metals in pure solution which could enhance the dissociation of acid in the DMEM. Interestingly, the solubility of Cd in the presence of As or Pb in UBM-DMEM solution seemed to fall in the same predictive line with that of single Cd (Figure 5.2). The solubility of As in UBM-DMEM solution was not influenced by the existence of Cd or Pb (Table S2 in Appendix 5), which indicates the insoluble compounds such as lead arsenate or cadmium arsenate were not produced when As, Cd and Pb co-existed in the cell culture medium. The possible reasons were suspected to be the same as discussed in Section 3.3.4: concentrations of free Cd/Pb cations and As anion were lower than the solubility (Ksp) of cadmium/lead arsenate.

The investigation of solubility of As, Cd and Pb in UBM-DMEM solution provides key information for *in vitro* cell studies. It points out that speciation should be taken into consideration since chemical reaction such as precipitation would significantly change the soluble concentrations and active forms of metal/metalloids (Twiss et al., 2001). This solubility problem of Pb puts the traditional toxicity studies using pure compounds as reported in the literature into question (Tchounwou et al., 2004). It is therefore strongly suggested that the solubility of metal/metalloids in the cell culture medium should be checked before carrying out cell exposure experiment.

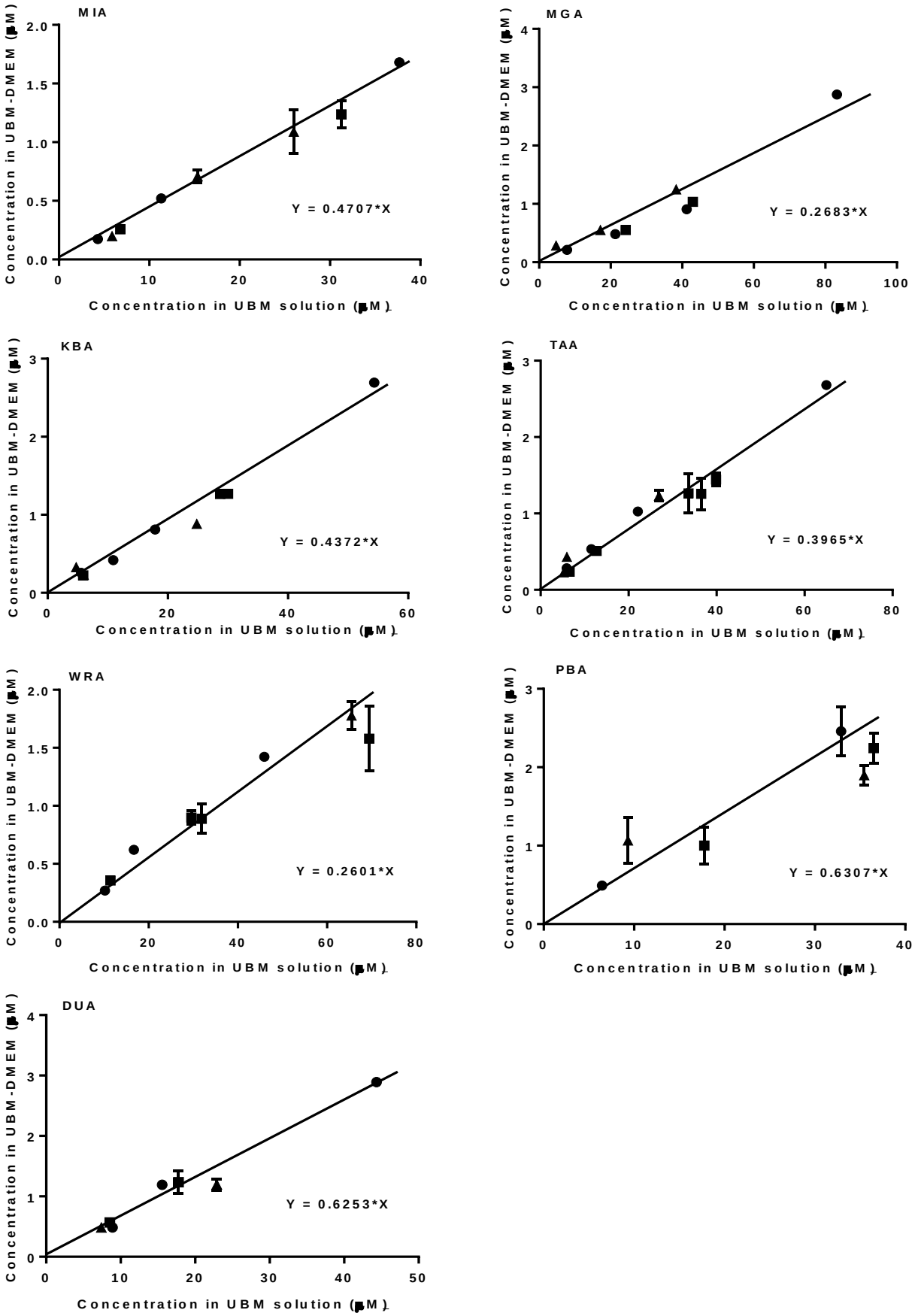

Figure 5.2 Linear regression analysis between concentrations of Cd in UBM solutions and those in UBM-DMEM solutions (v:v, 1:9). "●", "■", and "▲", represent solutions containing Cd, Cd+As and Cd+Pb, respectively. *p* values of all regressions were <0.0001. Each data is mean of triplicate or duplicate measurements ± standard deviation (SD).

5.3.2 Uptake of UBM-extracted As, Cd and Pb into HepG2 cells

Figure 5.3 illustrates the uptake of UBM-extracted As, Cd and Pb from seven types of soils in HepG2 cells after 24 hours of exposure. The uptake of Cd increased with the increase in the dosing concentrations, ranging from 1.84±0.8 to 10.9±2.0 ng/10^5 cells. However, an inflexion in the uptake was observed at higher concentrations of Cd which probably resulted from the increased toxicity at high dosing levels. Also, uptake at high concentrations showed higher variation (error bars shown in Figure 5.3) than those at low doses. Possible reason could be that when using PBS with 2 mmol L^{-1} EDTA to wash cells before harvesting, cells dosed with high levels of Cd were in a more damaged condition and may be sloshed off from the bottoms of the plate, which introduced uncontrollable systematic errors. It is noted that there was no significant difference in the uptake of Cd extracted from seven types of soils, which indicates that soluble Cd in UBM-DMEM solution was probably of the same species thus acted similarly. However, compared to the uptake of pure Cd, the uptake of UBM-extracted Cd was higher, which means that components in UBM-DMEM solution may facilitate Cd being absorbed into cells. Cadmium can be transported into cells via simple diffusion, ion channels and membrane carries, depending on the chemical forms and the abundance of Cd in the solution (Dawson and Ballatori, 1995). According to Pearson's classification (Pearson, 1963), Cd cation is a soft Lewis acid thus has a high affinity to easily oxidised soft ligands such as thiol-containing proteins or amino acids (Dawson and Ballatori, 1995). In UBM digestive fluids, bovine serum albumin (BSA), mucin, pepsin or their breakdown products (e.g. peptide, amino acids) can provide thiol groups to combine Cd. The thiol-bound Cd could be absorbed into cells via membrane carriers for amino acids, proteins or by endocytosis. In other words, proteins or their breakdown products in UBM solutions may provide another transport route for Cd thereby promote the uptake of Cd. DelRaso et al. (2003) found that the internalisation rate of Cd^{2+} in rat hepatocytes was enhanced by BSA because albumin-binding Cd increased the concentration of free ligands near cell membrane surface. Concentration of free ligands near the membrane surface was crucial for the rate of Cd transport through the membrane. Taken together, the existence of thiol groups was the key factor which increased the uptake of UBM-extracted Cd compared with pure Cd.

A dose-dependent manner was also observed for uptake of UBM-extracted As. However, unlike Cd exhibiting non-linear relationship between cell uptake and dosing concentrations,

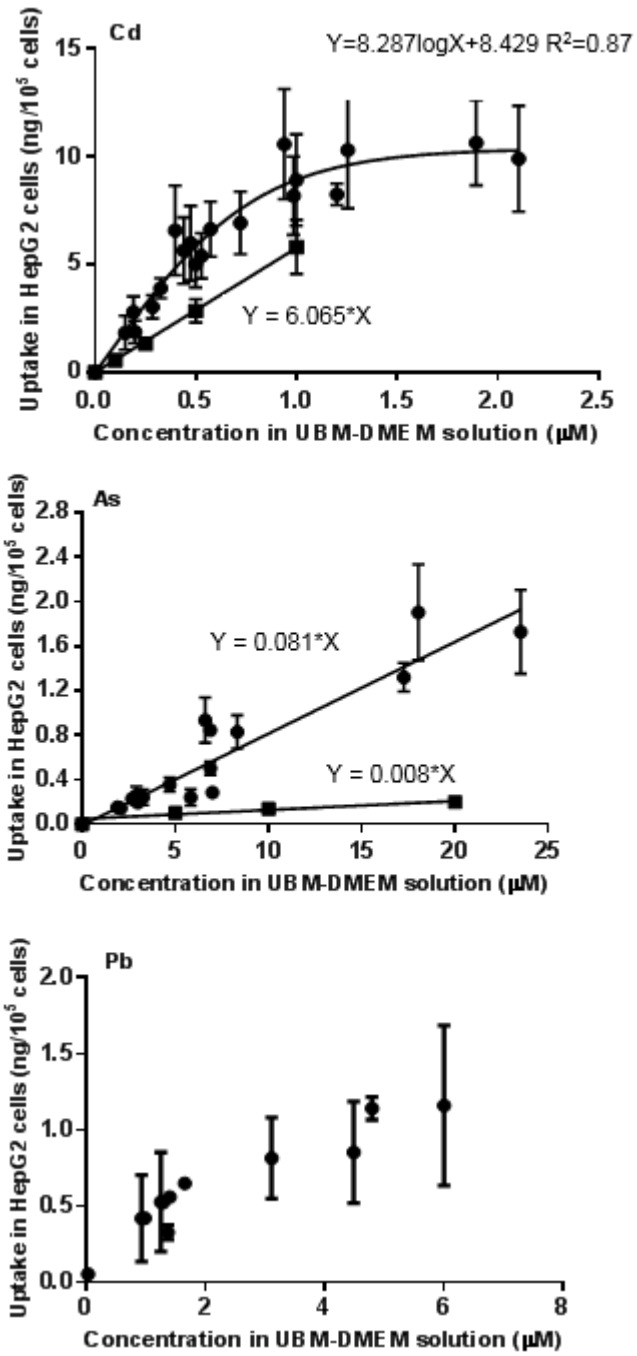

Figure 5.3 Uptake of UBM-extracted and pure As, Cd and Pb in HepG2 cells. "●" represents uptake of UBM-extracted As, Cd, or Pb and "■" represents the corresponding uptake of As or Cd in pure solution. Data are mean of at least triplicate measurements ± standard deviation (SD).

uptake of As seemed to have a linear relationship with As concentrations in UBM-DMEM solution. This may be accounted to extracellular As was far from the toxic concentration (Muthusamy et al. (2016) reported LC_{50} of As (Ⅲ) for HepG2 cells=159 μmol L^{-1}, As (V) is usually less toxic than As (Ⅲ)). Uptake values of As ranged from 0.14 ±0.017 to 2.07±0.50 ng/10^5 cell, which were significantly lower than those of Cd. It is well known that As (V), having a similar atomic structure as phosphate, is taken up by phosphate transporters in mammals (Rosen and Liu, 2009; Zangi and Filella, 2012). In UBM-DMEM solutions, the mean of phosphate concentrations was 0.98 mmol L^{-1} which was at least two orders of magnitude higher than those of As (1.90 to 23.52 μmol L^{-1}). Therefore, the limited uptake of As can be ascribed to the competition between As and phosphate for transporters. This phenomenon could happen under physiological condition since the concentration of phosphate in this cell culture system was similar to that in human plasma, 0.97 to 1.45 mmol L^{-1} (Dopp et al., 2010). Similar to Cd, the uptake of UBM-extracted As was much greater than that of pure As. In human liver system, As (V) was absorbed into cells and reduced to As (Ⅲ) which was then excreted from cells (Radabaugh and Aposhian, 2000; Drobna et al., 2010). The effusive As (Ⅲ) may bind thiol groups of protein in UBM-DMEM solution (Shen et al., 2013) and be absorbed into cell via amino acid or protein transporters. Due to the continuing precipitation phenomenon of Pb in UBM-DMEM solutions, limited data with relatively high variations was obtained for the uptake of UBM-extracted Pb (Figure 5.3). For the same reason, uptake data of pure Pb were not available because concentrations of Pb were under the detection limit for the exposure concentrations tested. Apparently, HepG2 cells cultured with DMEM was not a suitable system to investigate the uptake or toxicity of Pb due to the presence of high levels of phosphate and carbonate in the medium but can be a useful *in-vitro* model to predict the uptake of Cd and As under physiological conditions. It might be worthwhile to test the suitability of other cell culture medium containing relatively low concentrations of phosphate and carbonate.

5.3.3 Interaction effects of As, Cd and Pb on respective uptake into HepG2 cells

Interactions happened among As, Cd and Pb during uptake in HepG2 cells are summarised in Figures 5.4 and 5.5. It shows that the uptake of Cd was decreased when Cd co-existed with

As or Pb whilst the uptake of As was not affected by either Cd or Pb. For Pb, the results were ambiguous since the variations of some data were bigger than the corresponding means (data not shown). Throughout the literature, no study has reported the uptake interaction between binary mixtures of As, Cd and Pb in hepatocytes. Previous interaction data regarding the hepatic accumulation showed that As either decreased or had no effect on hepatic level of Cd whilst Cd was consistently reported to have no influence on As hepatic accumulation (Mahaffey et al., 1981; Elsenhans et al., 1987; Yanez et al., 1991). Even though our study utilised *in vitro* cell system, it was paralleled with *in vivo* results. For Cd-Pb interaction, additive, antagonistic and synergistic effects were reported with additive and antagonistic effects were mainly observed as summarised by ATSDR (2004). In this chapter, the inhibitory effect of Pb on Cd uptake in HepG2 cells seemed to be in agreement with some animal data mentioned in the ATSDR document. Hepatic levels of As and Pb were not affected by the existence of each other as reported in *in vivo* studies (Fairhall and Miller, 1941; Mahaffey et al., 1981; Elsenhans et al., 1987), which was also observed in our study. Interaction studies were also carried out for pure As, Cd and Pb. Except encountering the same precipitation problem for Pb, the interactions between As and Cd showed the same patterns as UBM-extracted As and Cd (Figure S1 in Appendix 5).

To sum up, even though *in vivo* studies came to varying conclusions regarding the interaction between binary mixtures of As, Cd and Pb in hepatic accumulation, additive or less than additive effects were most frequently reported. When *in vitro* liver cell system was adopted to investigate uptake interaction in hepatocyte in this chapter, results pointed similar trends as *in vivo* studies, which suggests HepG2 cells could be a promising cell-based tool to mimic physiological response in *in vivo* liver tissue. However, as discussed above, the *in vitro* model might not be suitable for Pb considering the precipitation problem. Another provoking finding is that despite As, Cd and Pb dissolved from soils did not interact with each other in simulated digestive system as concluded in Chapters 2 and 3, interaction may take place during subsequent uptake into target organ cell, such as liver cells. This observation is also an additional piece of evidence, suggesting the inconsistency in direction of interaction across different physiological processes (ATSDR, 2004). Therefore, great uncertainty could be expected when extrapolating interactions from one process (e.g. digestive system) to another (e.g. liver accumulation). It is beyond the scope of this study to establish a universal *in vitro* model but may provide useful information for interaction studies at hepatic level.

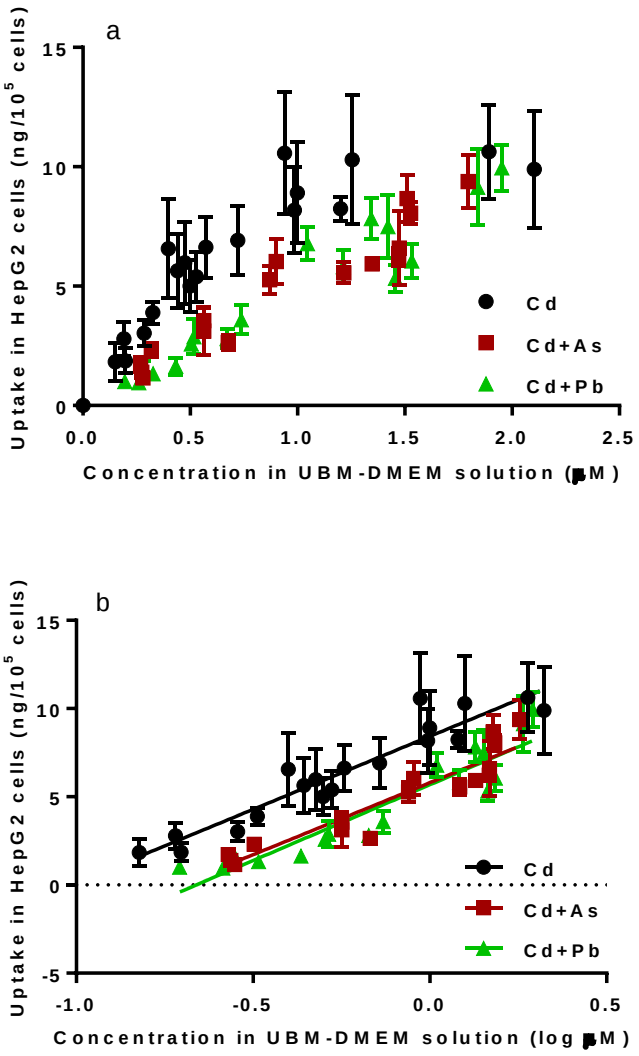

Figure 5.4 Effects of As and Pb on the uptake of UBM-extracted Cd. In Figure 4a, unit of X axis is Cd concentration in UBM-DMEM solution. Figure 4b, unit of X axis is log of Cd concentration in UBM-DMEM solution. Line of Cd (Y = 8.287*X + 8.429) in Figure 4b is significantly (p<0.0001) different from those of Cd+As (Y = 8.167*X + 5.799) and Cd+Pb (Y = 8.595*X + 5.681). Each data is mean of at least triplicate measurements ± standard deviation (SD).

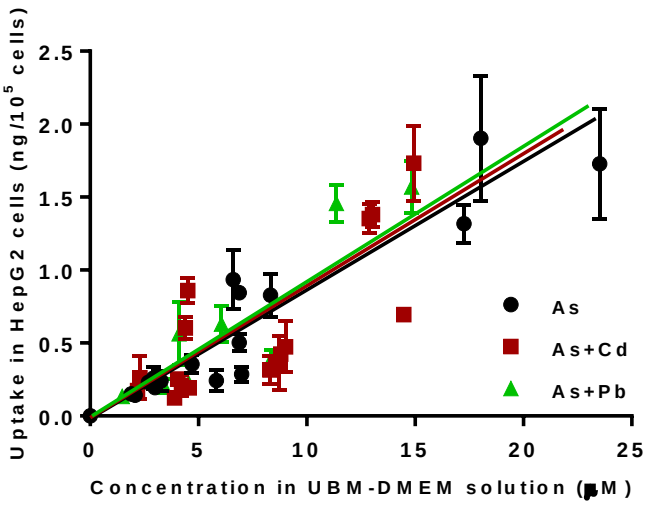

Figure 5.5 Effects of Cd and Pb on the uptake of UBM-extracted As. Lines "As+Cd" (Y=0.081X) and "As+Pb" (Y=0.082X) were not significantly different from line "As" (Y=0.081X). Each data is mean of at least triplicate measurements ± standard deviation (SD).

5.4 Conclusion

UBM-extracted Cd formed precipitate in DMEM but Cd solubility can be predicted on a soil-specific case. UBM-extracted As was stable at the initial concentration whilst most UBM-extracted Pb precipitated out in DMEM. The uptake of UBM-extracted As and Cd in HepG2 cells followed a dose-response relationship with Cd showing higher uptake than As. Interaction effects of As, Cd and Pb on respective uptake in HepG2 cell demonstrate that As and Pb can decrease the uptake of Cd whilst As uptake was not affected by either Cd or Pb, which did not conflict with *in vivo* data. Uptake of pure Cd and As was lower than that of UBM-extracted Cd and As. Interaction results of pure solution As and Cd exhibited the same pattern with UBM-extracted As and Cd. HepG2 cells maybe a promising *in vitro* cell system to predict uptake interaction of As and Cd under physiological condition but not suitable for Pb. Speciation should be taken consideration in *in vitro* studies, especially for Pb. Interaction patterns of mixed contaminants may not be consistent across different physiological processes. In future work, soils which have been put through bioavailability animal model could be of great value to ultimately correlate animal data with *in vitro* hepatic cell uptake data.

Chapter 6 Effects of PAHs on the bioaccessibility of arsenic, cadmium and lead as well as on the uptake into HepG2 cells

Abstract

PAHs (NAP, PHE, PYR and B[a]P) may co-exist with As, Cd and Pb at contaminated sites. However, no study has illustrated the potential effects of PAHs on the bioaccessibility of As, Cd and Pb as well their possible interaction during accumulation in target organ. Results show that losses in concentrations of PYR and B[a]P were significantly less than those in NAP and PHE when PAHs were aged in soils for up to 90 days. Bioaccessibility of As, Cd and Pb were not affected by PYR or B[a]P under two exposure scenarios. However, after UBM-extracted As, Cd and Pb progressed to target organ cells (HepG2), uptake of Cd was inhibited by PYR or B[a]P probably due to the more damaged cell membrane whilst uptake of As and Pb was not influenced. Pure solution results were in agreement with UBM-extracted mixtures. Taken together, bioaccessibility of As, Cd and Pb was not likely to be affected by PAHs due to distinct behaviours of inorganic and organic contaminants in soils and solutions whilst accumulation of metal/metalloid at toxic concentrations (e.g. Cd in this study) in hepatocytes might be inhibited by PAHs. This chapter provides crucial direction with regard to possible interaction among metal/metalloid and PAHs.

6.1 Introduction

Characterization of soil contaminated with mixtures of metal/metalloids has been widely reported in the literature. However, metal/metalloids may co-occur with organic substances at contaminated sites. For example, PAHs have been found as co-contaminants with metal/metalloids due to similar sources of contamination or materials and facilities used on the site. For examples, a strong correlation between metals (Pb, Cu, Zn) and PAHs in urban soils was obtained in previous studies, which can be attributed to automobile exhaust (Hwang and Cutright, 2002; Wang et al., 2004; Hofman et al., 2008; Morillo et al., 2008). Former gasworks sites were shown to be subjected to not only PAHs but also heavy metal contamination (Thavamani et al., 2011). Even in agricultural soils, relatively high correlation was found between PAHs and Pb concentrations (MaliszewskaKordybach et al., 1995). Therefore, the co-occurrence of metal/metalloids and PAHs is not unusual in the environment. However, most of published studies on risk assessment of contaminated sites have been focused on either metal/metalloids or organic pollutants without considering the possible interaction among mixed metal/metalloids and PAHs. Previous study pointed out that Cu and Al at high levels can occupy organic sorption sites in soils, thereby inhibiting the sorption of phenanthrene in soils and making it more labile (Obuekwe and Semple, 2013). Cadmium was reported to inhibit the dissipation of available benzo[a]pyrene in soil (Wang et al., 2014). The potential interaction between metal/metalloids and PAHs could lead to the uncertainty in risk assessment of contaminated soils, for instance in the evaluation of bioaccessibility. Previous studies have mainly reported the influence of metal/metalloids on PAHs whilst little attention has been paid to the potential impact of PAHs on the bioaccessibility of metal/metalloids. Chapters 2-4 have discussed the interaction effects of As, Cd and Pb on their respective bioaccessibility whilst this chapter would put emphasis on the effects of PAHs on the bioaccessibility of As, Cd and Pb. There is a great number of PAHs among which those molecules containing two to seven benzene rings are of environmental significance (Weast, 1969). Based on their frequency and toxicity, naphthalene (NAP), phenanthrene (PHE), pyrene (PYR) and benzo[a]pyrene (B[a]P) are selected as representatives of PAHs family to be investigated. These PAHs are 4 out of 16 priority PAH pollutants listed by U.S. EPA and are regarded as model PAHs (Gan et al., 2009).

PAHs in this study are specifically referred to as NAP, PHE, PYR and B[a]P. A variety of *in vitro* bioaccessibility systems has been developed for PAHs as summarised (Grøn, 2003).

Since the aim of this study is to examine the potential effects of PAHs on the bioaccessibility of As, Cd and Pb rather than vice versa, the United BARGE Method (UBM) was utilised here in order to keep consistent with Chapters 2-4. The interactive activities between As/Cd/Pb and PAHs were also examined at hepatic level by dosing HepG2 cells with UBM-extracted solutions containing mixtures of As/Cd/Pb and PAHs as methods described in Chapter 5.
To sum up, in this chapter, two types of soils were spiked with single or binary mixtures of As/Cd/Pb and PAHs. Bioaccessibility of As, Cd and Pb was measured at different ageing times (up to 90 days). The interaction effects of PAHs on uptake of As, Cd and Pb in HepG2 cells were followed on to be characterised. This chapter would shed light on the bioaccessibility of As, Cd and Pb when mixed with PAHs as well as explore the potential of HepG2 cells being a useful *in vitro* model to investigate the effects of PAHs on the hepatic accumulation of As, Cd and Pb.

6.2 Materials and methods

6.2.1 Soil sampling and characterisation

Seven variant soils were collected from Victoria and South Australia, namely Dublin (DUA), Kersbrook (KBA), Millicent (MIA), Mount Gambier (MGA), Port Broughton (PBA), Tarrington (TAA) and Wallaroo (WRA). Details can be found in Chapters 2 and 3 regarding soil sampling, processing, storage and characterisation for physio-chemical properties. Of these seven types of soils, soils collected from DUA and KBA were selected to carry out the As/Cd/Pb and PAH interaction study. The selection criterion was based on the differences in soil pH, total organic carbon and clay which were reported as important factors affecting bioaccessibility of PAHs (Luo et al., 2008; Badea et al., 2013). Table 6.1 presents key soil properties of KBA and DUA.

Table 6.1 Selected soil properties of KBA and DUA

Soil code	KBA	DUA
pH	4.45	7.31
Clay (%)	20	7.5
Silt (%)	37.5	12.8
Sand (%)	42.5	79.8
USDA texture*	Loam	loamy sand
TC (%)	5.5	1.6
TOC (%)	5.5	1.48
TIC (%)	0	0.12
DOC (mg kg^{-1})	400	213
CEC(cmol kg^{-1})	4.3	2.9
Fe oxide (g kg^{-1})	1.72	0.64
Al oxide (g kg^{-1})	2.13	0.74
Mn oxide (g kg^{-1})	0.0449	0.133
Total Fe (g kg^{-1})	22.65	9.87
Total Al (g kg^{-1})	11.13	7.98
Total Mn (g kg^{-1})	0.06	0.17

* USDA, the United States Department of Agriculture soil classification; Data represent the mean of duplicate analysis. Values varied by less than 5%.

6.2.2 Temporal change in total concentrations of PAHs in soils

After being air-dried and sieved to ≤ 2 mm, soils were spiked with NAP, PHE, PYR and B[a]P. Spiking chemicals were obtained from Sigma-Aldrich, Australia. The initial spiked concentrations were 1000, 1000, 1000 and 200 mg kg^{-1} for NAP, PHE, PYR and B[a]P, respectively. PAHs were dissolved in hexane and added drop-wise to soils. After PAHs dispersed in soils, solvent was allowed to volatilise in the fume hood in order to minimise the effect of solvent on soils (Northcott and Jones, 2000). Soils were freeze-dried and measured for concentrations after 7, 30 and 90 days of ageing at ambient temperature. Concentrations of PAHs were measured by GC-MSMS (Agilent 7890A, Japan) and LC-MSMS (Thermo Scientific Q-Exactive, Australia). The LODs were 1 µg L^{-1} for NAP, PHE, PYR and 5 µg L^{-1} for B[a]P. Laboratory background of PAHs was not detected. Laboratory blanks and a reference material (European Commission BCR® -524 - industrial soil) were included and recoveries of internal and surrogate standards were used as a measure of QA/QC in the

procedures. Briefly, approximately 0.1 g of spiked sample was extracted using a mixture of acetone/hexane (1:1 v/v, 2 mL). Then 0.1 g of the AOAC 2007.01 extraction salt (containing magnesium sulfate and sodium acetate) was introduced and the contents were vortexed for 5 min for further extraction. This was followed by centrifuging the extract for 5 min at 3000 g. Then 1 mL of the supernatant was transferred to the dispersive solid phase extraction (d-SPE) tube and cleaned up by vortexing the contents for 5 min, followed by centrifuging at 13,500 g for 5 min. The clean extract was ready for analysis of PAHs by GCMS/MS (Gas Chromatography Mass Spectrometer). A 30 m x 0.25 mm ID and 0.25 µm DB-5MS column was used with helium as carrier gas (flow rate = 1.2 mL min^{-1}). Injector temperature was maintained at 270℃ while the oven temperature was programmed with an initial temperature of 60℃, ramped to 210 ℃ at 120C min^{-1} then 80 C min^{-1} to 340 ℃ with 5 min hold time.

6.2.3 Soil amendment with As, Cd, Pb and PAHs

PAHs amended in soils for interaction study were PYR and B[a]P based on the stability of concentrations during ageing (discussed in Section 6.3.1). Metals/metalloid chemicals were purchased from Sigma-Aldrich, including As (sodium arsenate), Cd (cadmium nitrate) and Pb (lead nitrate). After being air-dried and sieved under 2 mm, soils were spiked with single As, Cd, Pb, PYR and B[a]P. Spiking procedure for As/Cd/Pb was described in Section 2.2.3 (pages 46-47). PAHs were spiked as mentioned above. Soils spiked with single contaminant were aged for one year at ambient temperature to mimic independent ageing of contaminants as soil samples discussed in Chapters 2 and 3. The maintenance of soil samples during ageing was described in Section 2.2.2 (page 45).

Except for soils spiked with single contaminant, soils were also spiked with binary mixtures of As/Cd/Pb and PAHs (As+PYR, As+B[a]P, Cd+PYR, Cd+B[a]P, Pb+PYR or Pb+B[a]P) to simulate mixed contaminants ageing together in the same soil as described in Chapter 4. Arsenic, Cd or Pb was first introduced to soils individually followed by 24 hours of sorption. Thereafter, soils were freeze-dried and PAHs were added into soils (hexane was added to control soils spiked with single As, Cd or Pb) followed by evaporation of solvent. The maintenance of soil samples during ageing was described in Section 2.2.2 (page 45).

6.2.4 Bioaccessibility measurement

Prior to bioaccessibility measurement, total concentrations of As, Cd and Pb in sieved soils were determined by ICP-MS after microwave-assisted acid digestion (EPA, 2007). Ingredients of UBM fluids and procedures were available in Appendix 1. For independently-aged soil samples spiked with single As, Cd, Pb or PAH, equal portions (0.15 g) of metals/metalloid-spiked (As/Cd/Pb) soil and PAH-spiked (PYR/B[a]P) soil were weighed sequentially into the same container and extracted in UBM fluids. For simultaneously-aged soil samples spiked with mixtures of As/Cd/Pb and PAHs, subsamples were analysed for bioaccessibility at the time points of 7, 30, 90 days. Bioaccessibility which is expressed in percentage (%) refers to the ratio between bioaccessible metals/metalloid and total metals/metalloid concentration in soil (<250 μm). Both gastric bioaccessibility and intestinal bioaccessibility were reported.

6.2.5. Measurement of uptake in HepG2 cells

HepG2 (HB-8065, ATCC, U.S.) cells were adopted to investigate the uptake interaction between As/Cd/Pb and PAHs. The maintenance of cell culture, preparation of dosing solutions and quantification of As/Cd/Pb uptake were described in Materials and Methods section of Chapter 5 (pages 91-92). UBM gastric-phase solutions containing binary mixtures of As/Cd/Pb and PYR/B[a]P extracted from independently-aged soils (Chapters 2 and 3) were utilised for uptake study.

6.2.6 Quality assurance (QA) and quality control (QC)

Please refer to Section 3.2.4 (page 62) for details.

6.2.7 Statistical analysis

Statistical analysis methods regarding linear regression and comparison were described in Section 2.2.5 (page 48).

6.3 Results and discussion

6.3.1 Temporal change in soil PAH concentrations

The variations in total PAH concentrations in soils with time were shown in Table 6.2. The dissipation of four PAHs was observed in both types of soils, which can be attributed to microbial degradation or abiotic losses such as photodegradation and volatilization (Wang et al., 2014). NAP was reduced to 2 mg kg^{-1} at the time point of 30 days presumably because of its high volatility and rapid degradation (Kastner and Mahro, 1996; El-Masri, 2005). Average losses in concentrations of PHE, PYR and B[a]P were 65%, 35% and 45% in KBA soils and 58%, 35% and 52% in DUA soils, respectively. The decline in total concentrations of PYR and B[a]P was smaller than that of PHE due to the increased hydrophobicity and resistance to microbial degradation with increased aromatic rings (Kanaly and Harayama, 2000). Throughout the literature, there was a lack of consistency in the loss of PAHs in spiked soils with time. For example, over 50% losses in total PHE and PYR were observed by (Šmídová et al., 2012; Obuekwe and Semple, 2013) in 56 or 63 days whilst less than 13% dissipation was reported by Hwang and Cutright (2002) in 200 days of ageing. The inconsistency can be explained by (1) different methods utilised to measure organic contaminants: for instance, ^{14}C-analysis not only measures parent compounds but also degraded products whilst the GC/MS instrumental analysis only calculates parental compounds (Šmídová et al., 2012); (2) sterilisation: Contreras-Ramos et al. (2006) reported that the reduction in PHE in natural soils aged for 11 weeks was as high as 88% but only 8-20% in sterile soils; (3) spiked concentrations: catabolic activities might be triggered when PAH concentrations were above threshold values (Macleod and Semple, 2000); and (4) spiking procedures including spiking method, carrier solvent, mixing, homogeneity and equilibrium: e.g. carrier solvent has the potential to affect organic matter in soils where the sorption of PAHs mainly takes place (Northcott and Jones, 2000). Based on the stability of concentrations in soils, PYR and B[a]P were chosen for interaction study with As, Cd and Pb.

Table 6.2 Temporal variations in the total PAHs in KBA and DUA soils

	Concentration (mg kg^{-1}) in KBA soils				Concentration (mg kg^{-1}) in DUA soils			
Ageing time (d)	NAP	PHE	PYR	B[a]P	NAP	PHE	PYR	B[a]P
7	119	837	966	195	138	880	1101	184
30	2	408	683	113	2	422	683	100
90	2	353	651	110	2	416	651	96

Data represent mean of duplicate analysis.

6.3.2 Effects of PYR and B[a]P on the bioaccessibility of As, Cd and Pb in separately-aged soils

Soils were spiked with single contaminant, including As, Cd, Pb, PYR, B[a]P and aged for one year. Equal proportions of soils spiked with As/Cd/Pb or PYR/B[a]P were added into the same container for bioaccessibility extraction with the aim to mimic the simultaneous ingestion of mixed contaminants which were aged independently, e.g. children ingest soil containing As/Cd/Pb at one spot prior to ingestion of PAH-bearing soil at another spot or soils possessing multiple contaminants which enter the same spot at long time intervals. Results (Table 6.3) demonstrate that bioaccessibility of As, Cd and Pb were not affected by the co-existing PYR or B[a]P in the simulated digestive fluids (p>0.05). These results are in accordance with what was observed in Chapters 2 and 3, which indicates independent ageing enabled each metal/metalloid to establish its own relationship with soil functional groups. Thus no significant interaction was obtained when interaction studies were carried out by loading two aliquots of the same type of soil spiked with As/Cd/Pb or PYR/B[a]P in one extraction container. In the simulated digestive fluid, bile has been reported to act as surfactant which can decrease the surface tension of digestive solution and dissolve more PAHs from soils by forming bile micelle (Tang et al., 2006b). Also, bile can complex with metal/metalloids (Feroci et al., 1995). It seems that PYR and B[a]P did not compete against As/Cd/Pb for binding sites of bile probably due to adequate concentration of bile (>1000 mg L^{-1}).

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Table 6.3 Effects of PYR and B[a]P on the bioaccessibility of As, Cd and Pb in spiked soils (independent ageing)

Soil code	As bioaccessibility			Cd bioaccessibility			Pb bioaccessibility		
	Total As (mg kg^{-1})[a]	G (%)[b]	GI (%)[b]	Total Cd (mg kg^{-1})[a]	G (%)[b]	GI (%)[b]	Total Pb (mg kg^{-1})[a]	G (%)[b]	GI (%)[b]
KBA	80	68±3.4	68±2.8	61	74±1.6	20±4.5	569	69±1.8	18±2.5
	80 (PYR)	65±1.2	64±1.6	61 (PYR)	74±2.0	19±0.5	569 (PYR)	65±1.6	18±0.5
	80 (B[a]P)	69±0.9	67±1.1	61 (B[a]P)	77±0.3	21±1.3	569 (B[a]P)	68±0.8	17±4.3
DUA	101	83±9.3	73±11.2	75	90±5.7	41±0.6	443	78±12.9	35±1.7
	101 (PYR)	82±0.1	73±0.1	75 (PYR)	92±1.7	41±1.0	443 (PYR)	83±0.4	33±1.4
	101 (B[a]P)	82±0.9	75±0.7	75 (B[a]P)	91±0.7	39±0.1	443 (B[a]P)	80±6.5	32±4.7

[a] Data represent mean of duplicate analysis. [b] Data represent mean of triplicate measurements + standard deviation (SD). "G" stands for gastric bioaccessibility; "GI" represents intestinal bioaccessibility. "As bioaccessibility, 80 (PYR)" means 0.15 g soil spiked with 80 mg kg^{-1} As was mixed with 0.15 g soil spiked with PYR, etc. Bioaccessibility of As, Cd and Pb were not affected by the co-existing PYR or B[a]P in the digestive system(p>0.05).

6.3.3 Effects of PYR and B[a]P on the bioaccessibility of As, Cd and Pb in simultaneously-aged soils

Binary mixtures of As/Cd/Pb and PYR/B[a]P (As+PYR, As+B[a]P, Cd+PYR, Cd+B[a]P, Pb+PYR or Pb+B[a]P) were added to KBA or TAA soils in order to simulate the scenario where contaminants enter the same spot concurrently and age simultaneously. Bioaccessibility of As, Cd and Pb was analysed post ageing time of 7, 30 and 90 days and results are summarised in Table 6.4. As can be seen from the table, during this period time of ageing, the occurrence of PYR or B[a]P in soils did not show effects on the solubility of As/Cd/Pb in digestive system (p>0.05). In a previous study, the partitioning of Cd to soil fractions (soluble Cd, exchangeable Cd, carbonate-bound Cd, Fe/Mn oxides-bound Cd, organic-bound Cd and residual Cd fractions) in Cd sole-polluted soils was not significantly different from that in Cd-PYR co-contaminated soils (Zhang et al., 2009). Bioaccessible Cd mainly derived from the soluble and exchangeable fractions in soils (Tang et al., 2006a) thereby it is postulated that bioaccessibility of Cd may stay unchanged in the presence of PYR despite this was not measured by Zhang et al. Based on this observation, it was not surprising that no interaction was detected in the current study. In addition, it has been reported that organic matter provides important reservoir for PAHs (Means et al., 1980; Xing, 2001) in soils as well as exhibits strong affinity for metal/metalloids (Takamatsu et al., 1983; Warwick et al., 2005). It seems that PYR/B[a]P did not interfere with the sorption of As/Cd/Pb to organic carbon due to the distinct mechanism of sorption or low levels of contaminants in soils. In reviewing the literature, the influences of metal/metalloids on the behaviours of PAHs in soils (e.g. sorption, partitioning, dissipation, bioaccessibility) attract more attention than vice versa (Gao et al., 2006; Obuekwe and Semple, 2013; Wang et al., 2014). For example, the sorption of PHE was enhanced by heavy metal cations (e.g. Cu^{2+}. Pb^{2+}) because rubbery organic carbon in soils (including flexible dissolved organic carbon and humic acids) was condensed on solid surfaces in the presence of metal cations (Luo et al., 2010). Cadmium inhibited the dissipation of available B[a]P due to the toxic effect of Cd on the indigenous soil microflora (Wang et al., 2014). However, very little has been reported regarding the influence of PAHs on metal/metalloids. Therefore, interaction between metal/metalloids and PAHs might not be reciprocal under some situations, e.g. in soil environment: metal/metalloid may affect the activities of PAHs in soils by impacting the microflora in soils whilst PAHs barely influence metal/metalloids.

Table 6.4 Temporal change in bioaccessibility of As, Cd and Pb with ageing time when soils were spiked with binary mixtures of As/Cd/Pb and PYR/B[a]P

Ageing time (d)	KBA						DUA					
	As		As+PYR		As+B[a]P		As		As+PYR		As+B[a]P	
	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)
7	87±1.7	83±4.2	84±1.2	77±0.3	92±3.1	74±6.1	92±1.1	83±0.4	89±4.0	83±1.6	92±5.6	86±3.1
30	62±8.3	68±2.0	69±1.0	69±2.3	71±1.4	70±1.9	86±1.6	80±0.8	86±0.6	81±0.8	85±0.5	79±0.4
90	67±0.7	54±6.5	64±1.5	50±0.9	68±0.7	52±0.5	84±0.1	79±10	84±0.8	66±12.7	81±1.8	70±7.3
	Cd		Cd+PYR		Cd+B[a]P		Cd		Cd+PYR		Cd+B[a]P	
	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)
7	83±1.8	22±1.9	88±1.3	24±2.9	87±0.1	21±1.2	93±0.8	34±4.8	94±0.1	32±6.6	84±0.2	31±2.2
30	84±1.3	18±0.1	87±0.8	19±0.6	83±0.1	18±1.7	93±6.0	37±4.2	90±2.1	37±0.6	87±3.2	33±3.8
90	85±1.1	25±1.0	87±0.9	25±0.2	90±4.1	26±0.2	90±2.4	37±1..3	90±1.3	32±3.2	92±0.5	36±1.2
	Pb		Pb+PYR		Pb+B[a]P		Pb		Pb+PYR		Pb+B[a]P	
	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)	G (%)	GI (%)
7	80±1.5	22±0.7	76±0.5	22±0.3	76±1.7	21±1.5	80±0.4	29±2.9	79±0.3	30±0.8	84±0.5	31±2.2
30	77±1.9	19±1.1	76±0.3	20±0.5	77±0.2	18±0.2	82±4.3	32±0.2	78±1.1	28±2.8	75±1.3	31±0.3
90	78±3.3	20±2.6	74±1.0	23±3.6	74±0.1	19±1.4	78±0.1	30±2.3	77±0.6	28±1.2	79±0.5	29±2.1

Data represent mean of triplicate measurements ± standard deviation (SD); Bioaccessibility of As/Cd/Pb in mixtures was not significantly different from that of single As/Cd/Pb at corresponding ageing time (p>0.05). "As+PYR" means soils were spiked with mixture of As and PYR; "As+B[a]P" means soils were spiked with mixture of As and PYR; "Cd+PYR" means soils were spiked with mixture of Cd and PYR; "Cd+B[a]P" means soils were spiked with mixture of Cd and PYR; "Pb+PYR" means soils were spiked with mixture of Pb and PYR; "Pb+B[a]P" means soils were spiked with mixture of Pb and PYR. Bioaccessibility of As, Cd and Pb were not affected by simultaneously aged PYR or B[a]P (p>0.05).

6.3.4 Effects of PYR and B[a]P on the uptake of As, Cd and Pb into HepG2 cells

The solubility of UBM-extracted As, Cd and Pb in cell culture medium was not affected by the co-occurring PYR or B[a]P and followed the same pattern discussed in Section 5.3.1 (Table S1 in Appendix 6). Most of UBM-extracted Pb was precipitated in the cell culture medium. Thus only uptake results of As and Cd mixed with PYR or B[a]P were illustrated in Figure 6.1. As can be seen from the figure, the uptake of As was not affected by PYR and B[a]P whilst the uptake of Cd was decreased by PYR and B[a]P. The reduction in Cd uptake at high concentrations (0.63/0.67 μmol L^{-1}) was more pronounced than that at low concentrations (0.28/0.29 μmol L^{-1}), which indicates interaction may be dose-dependent and toxicity-based. The same scenarios were observed for the uptake of As and Cd in pure solutions mixed with PYR or B[a]P (Figure S1 in Appendix 6). Inhibitory effects of PYR and B[a]P on the uptake of pure solution Cd was observed at dosing level of 0.5 μmol L^{-1}. Due to their distinct physiochemical properties (e.g. hydrophobic/hydrophilic), PYR and B[a]P would be not likely to interfere the uptake of Cd by competition. The possible reason could be the deleterious effects of PYR and B[a]P on membrane integrity and permeability to metals (Gauthier et al., 2014). Take uptake interaction results in Chapter 5 for comparison, it seems Cd was the only metal which suffered from effects of other compounds. Muthusamy et al. (2016) reported LC_{50} of Cd in HepG2 cells was 2.7 μmol L^{-1} whilst LC_{50} of As in HepG2 cells was >159 μmol L^{-1}. Concentrations of Cd in UBM-DMEM solutions were more close to its toxic dose than As did. Therefore, it could be possible that metal/metalloid close to toxic doses was more easily affected (inhibition) as a mechanism of protection. In addition, taken interaction studies of bioaccessibility and uptake together, it suggests that the interaction may vary with different physiological processes. In this study, interactive activities between Cd and PYR/B[a]P were not observed in simulated digestive system but PYR/B[a]P interfered the uptake of Cd into target organ cells (HepG2 cells here).

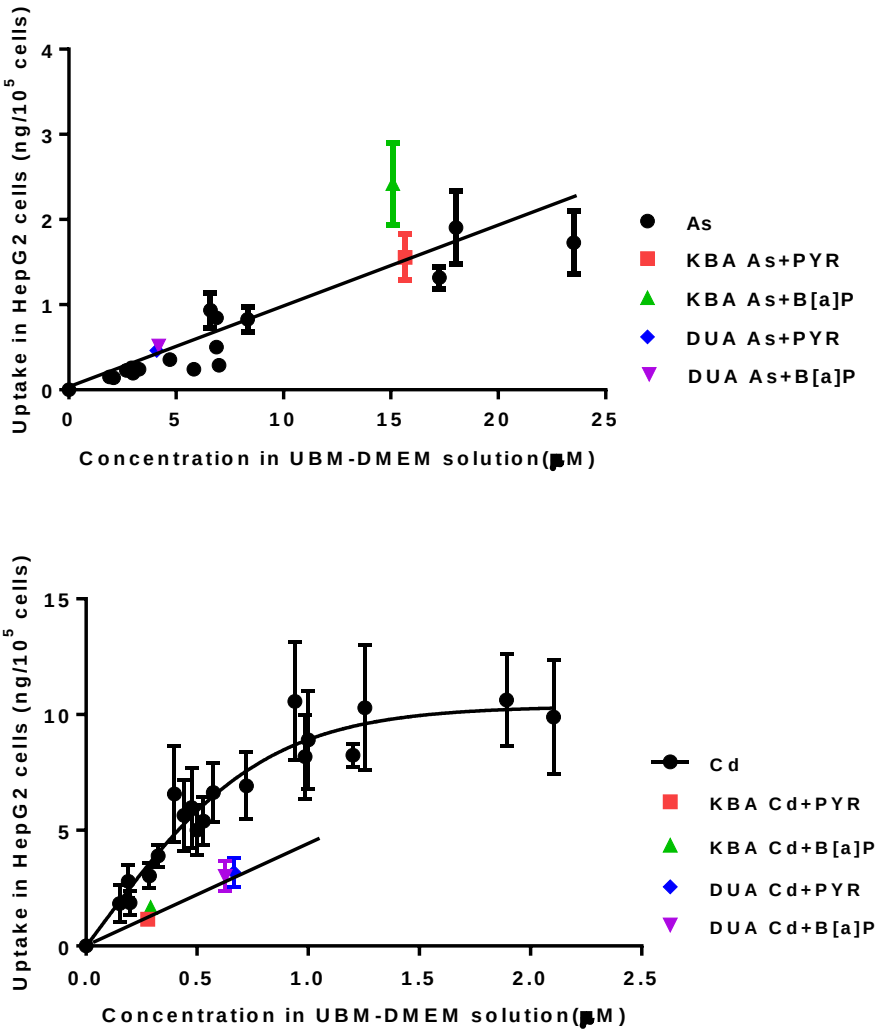

Figure 6.1 Effects of PYR/B[a]P on the uptake of As and Cd in HepG2 cells. Each data is mean ± standard deviation (SD)

6.4 Conclusion

Among NAP, PHE, PYR and B[a]P, PYR and B[a]P showed the lower losses in total concentrations in soils up to 90 days. Bioaccessibility of As, Cd and Pb were not affected by PYR or B[a]P in simulated digestive system whether soils were spiked with binary mixtures of As/Cd/Pb and PYR/B[a]P (simultaneously ageing) or soils spiked with single As/Cd/Pb was mixed up with soils spiked with PYR/B[a]P (independent ageing) during UBM extraction. Interestingly, the inhibiting effects of PYR and B[a]P on the uptake of Cd in HepG2 cells were observed, which indicates: (1) accumulation of metal/metalloid at adverse dose (e.g. Cd in this study) in hepatocytes might be suppressed by co-occurring PAHs; (2) interaction between As/Cd/Pb and PYR/B[a]P may be interpreted according to different physiological processes. This work provides a good opportunity to advance the understanding of mixed metal/metalloid and PAH contaminants and would add important knowledge to risk assessment associated with mixed metal/metalloid and PAHs.

Chapter 7 Final discussion, conclusions and future work

In this chapter, key results are summarised for a final discussion regarding soil-bioaccessibility relationships, bioaccessibility interaction, uptake interaction in HepG2 cells. Conclusions are made after discussion section. In the end, future work is suggested.

7.1 Final discussion

7.1.1 Bioaccessibility of As, Cd and Pb in spiked soils

Taking average of bioaccessibility data obtained from seven types of soils aged for one year (Chapters 2 and 3), bioaccessibility of As, Cd and Pb was 67, 85 and 80% in the gastric phase, and 61, 26 and 16% in the intestinal phase, respectively. It is noted that there was a significant decrease in bioaccessibility of Cd and Pb in the intestinal phase compared to that in the gastric phase whilst only slight decrease was observed in As bioaccessibility in the intestinal phase. Also, gastric bioaccessibility of Cd and Pb was much higher than that of As. These differences can be explained by the following reasons. Cadmium and Pb could be released from binding ligands such as oxides under strong acid environment in the UBM gastric fluid (pH 1.2). When soils moved to intestinal compartment (pH=5.5-6.8), re-adsorption on soil ligands, chemical precipitation and complexation by pepsin may take place, thereby reducing bioaccessibility significantly. By contrast, as a metalloid, As exists as an anion (e.g. $AsO4^{3-}$), would not undergo competition with H^{+} in the gastric phase and would not react with the anions (e.g. carbonate, sulphur, phosphate) to form chemical precipitation in the intestinal phase due to its anion state. When comparing bioaccessibility of two metals, Cd and Pb, bioaccessibility of Pb was lower than those of Cd, especially in the intestinal phase. This was not surprising considering Pb possesses greater hydrolysis constant, higher atomic weight and ionic radius, and larger Misono softness value over Cd, which favoured Pb to more readily undergo inner-sphere surface sorption and complexation

than Cd. In a summary, As was least mobile in the gastric phase whilst Pb was least mobile in the intestinal phase.

Furthermore, bioaccessibility of As, Cd and Pb measured in soils aged for three months (Chapter 4) was similar to that measured in soils aged for one year, which indicates that three-month ageing maybe sufficient for As/Cd/Pb to reach a relatively steady state in soils. The most significant reduction in bioaccessibility was completed for the first seven days of ageing, which demonstrates a rapid reaction between As/Cd/Pb and soil components under the ageing condition of this study.

Bioaccessibility data of spiked soils were also compared with data of real environmental samples reported in the literature. It is found that results of spiked soils were consistent with those of field soils on the condition that they shared similar physiochemical properties. This finding sheds light on the reliability of the current laboratory processing to represent field situations with the respect to bioaccessibility studies.

The validity of the UBM was further proved in the current study by the results of certified reference materials, NIST 2710A and 2711A as well as the repeatability of bioaccessibility result for individual soil sample. It is worthy of notice that more variations were observed for gastrointestinal bioaccessibility data than gastric bioaccessibility data despite the pH was controlled at the beginning and in between the gastric and intestinal phases during the UBM extraction. Perhaps, a better control and more frequent monitoring of pH in the intestinal phase, as well as better homogeneity of simulated intestinal solutions, may help to improve its repeatability and reproducibility.

7.1.2 Relationships between bioaccessibility, total concentrations and selected soil properties

Strong linear relationships between bioaccessible As/Cd/Pb (both gastric and intestinal phases) and total As/Cd/Pb in soils were observed with r^2 varying between 0.72 and 0.98 (p<0.0001). Goodness of fit between gastric bioaccessible As/Cd/Pb and total As/Cd/Pb in soils (r^2=0.92, 0.98, 0.96) was stronger than those between intestinal bioaccessible As/Cd/Pb and total As/Cd/Pb (r^2=0.72, 0.73, 0.85). Total organic carbon, Fe oxide, Al oxide were key parameters decreasing gastrointestinal bioaccessibility of As and Cd among which organic carbon was the most influential one based on values of r^2 (p<0.0001). However, these results of relationships can be misleading if only values of r^2 were taken into consideration. Take KBA and TAA for example: bioaccessibility values of As, Cd and Pb in TAA soils were found to be significantly lower than those in KBA soils (p<0.05). When comparing their soil properties, TAA soils possessed lower organic carbon (4.97%) but higher contents of Fe (3.12 g kg^{-1}) and Al oxides (3.01 g kg^{-1}) than KBA soils (organic carbon, 5.5%; Fe oxide, 1.72 g kg^{-1}; Al oxide, 2.13 g kg^{-1}). Another observation was that there were no significant relationships between Pb gastrointestinal bioaccessibility and organic carbon/Fe oxide/Al oxide (r^2=0.11-0.38). It was found that the gastrointestinal bioaccessibility of Pb in WRA soils was extremely low (1-2%) due to a great amount of carbonate in soils and these low values introduced significant deviation to the linear regression. Therefore, relationships between Pb gastrointestinal bioaccessibility and organic carbon/Fe oxide/Al oxide were plotted for the second time without results of WRA soils. r^2 values were dramatically increased to 0.41-0.43, which meant organic carbon, Fe oxide and Al oxide were also important factors controlling Pb gastrointestinal bioaccessibility. From what has been discussed above, it is suggested that interpretation of soil-bioaccessibility relationships should be taken with caution. Since only 7 types of soils are studied, limited samples might lead to coincidental correlation

without showing the real trend. More soil types would help to avoid this kind of uncertainty.

Another limitation for these relationships was that there was a strong correlation (Pearson r^2=0.9778, p<0.001) between contents of Fe oxide and contents of Al oxide in the seven types of soils. Thus the effect of Fe oxide on the bioaccessibility cannot be differentiated from that of Al oxide. This was considered as a coincidence which cannot be controlled at the stage of soil sampling. The coincidence might be avoided if more soil types can be included.

After comparing with published studies, we have found that the relationships between organic carbon/Fe oxide/Al oxide and bioaccessibility of As/Cd/Pb are consistent with published data but clay, as a widely-reported soil property that impacts bioaccessibility, was not observed to have any effect on bioaccessibility in the study. This indicates that bioaccessibility data might be site-specific and cannot be explained solely by soil properties but may be subject to a variety of factors such as speciation, mineralogy, ageing conditions, even bioaccessibility measurement methods. Especially for Pb bioaccessibility, mineralogy (carbonate in this study) has been observed as a crucial controller. However, this thesis was focused on the role of soil properties and limited by its sample size to explore the effect of mineralogy, therefore more soil types should be studied in the future to account for variation parameters such as mineralogy of Pb. Synchrotron-based X-ray Spectroscopy and XRD should be considered with the aim to identify Pb speciation/minerals in a wider range of soils. Nevertheless, soil-bioaccessibility relationships gained in this study add useful information to the Australian soil data and could be used as a base for comparison in future studies.

7.1.3 Interaction effects of As, Cd and Pb on their respective bioaccessibility

Two exposure scenarios were mimicked to investigate the interaction among soil-born As, Cd and Pb in the digestive system. Scenario one (Chapters 2 and 3): humans ingest independently-aged contaminants, e.g. contaminants are aged in different spots or there is a long time interval between different contaminants entering the same spot; and Scenario two (Chapter 4): humans take in simultaneously-aged contaminants, e.g. contaminants enter the same soil and are aged concurrently. Scenario one was simulated by mixing up two separate portions of the same type of soils spiked with one metal/metalloid or another metal/metalloid in the same extraction container. Scenario two was studied by spiking As/Cd/Pb in the same soil sample sequentially.

Results of Scenario one show that As, Cd and Pb did not impact each other's bioaccessibility in seven types of soils studied. Since As, Cd and Pb were spiked to individual soils of the same type, As, Cd and Pb would not interact with each other during ageing. When independently-aged As and Cd (or As and Pb) co-existed in the digestive fluids, insoluble compounds such as metal arsenate were unlikely to be produced for the reason that concentrations of free ions of As, Cd and Pb were lower than the solubility (Ksp) of cadmium/lead arsenate due to the binding of Cd/Pb to thiol groups of protein (e.g. bile, bovine serum albumin) in digestive fluids. When Cd and Pb co-occurred, no competition for binding ligands was observed considering concentrations of binding sites were much higher than solubilised Cd or Pb. Therefore, the interaction effects of independently-aged As, Cd and Pb on their respective bioaccessibility highly depend on the concentration levels of As, Cd and Pb in the UBM extraction system.

Results of Scenario two afford a different story from that of Scenario one. Four types of soils (MGA, KBA, TAA and DUA) were selected to be spiked with mixtures of As, Cd and Pb (As+Cd, As+Pb, Cd+Pb, Cd+Pb+As) sequentially at 24-hour interval. Bioaccessibility was measured at the ageing time of 7, 30 and 90 days. Gastric

bioaccessibility of As, Cd and Pb in all co-contaminated soils was not significantly different from that in soils which were individually spiked with As, Cd or Pb at corresponding ageing time (p>0.05). When soils moved from the gastric phase to intestinal phase, bioaccessibility of As, Cd and Pb in soils spiked with binary mixtures of As, Cd and Pb was not affected by the other co-existing metal/metalloid. However, when Pb and As were added to Cd-spiked soil one after another, intestinal bioaccessibility of Cd in KBA and TAA soils was increased compared with that in soils spiked with single Cd during early stage of ageing. In contrast, bioaccessibility of As and Pb was not influenced by Cd. Since no interaction among As, Cd and Pb was expected to take place during extraction because of concentration limit as discussed above, the increase in Cd intestinal bioaccessibility was assumed to result from interactive activities in soils, such as initial competition for sorption sites. As ageing continued, the bioaccessibility of Cd in soils spiked with As, Cd and Pb was back to the same level as that in soils spiked with single Cd. However, this interaction was not observed in another acidic soil, MGA soils, which may be attributed to the significant amounts of organic carbon, Fe oxide and Al oxide in this type of soil. Interestingly, even though DUA soils contained fewer sorption sites than other three types of soils, interaction effects of As and Pb on Cd intestinal bioaccessibility were not observed during ageing. It is postulated that under alkaline condition of DUA soils (pH=7.31), high pH may favour Cd to interact with soil components rapidly, thus leaving less chance to be affected by subsequent As and Pb. Taken together, the time for Cd to reach a steady intestinal bioaccessibility was prolonged at the presence of As and Pb in acidic soils with limiting binding sites. However, soils containing a large amount of organic carbon, Fe oxide and Al oxide or alkaline pH seemed to be free of the above-mentioned interaction.

We took a further step to investigate whether the time interval could affect results of interaction. Therefore, As and Pb were introduced to soils after Cd had been incubated in

soils for 7 days. Bioaccessibility of Cd was measured after soils were aged with ternary mixture of As, Cd and Pb for another 7 days. Results demonstrate no appreciable effects of As and Pb on intestinal bioaccessibility of Cd were detected in KBA and TAA soils. Since the most significant reduction in Cd bioaccessibility was observed within the first week, 7 days maybe sufficient for Cd to develop stable bindings to soils, thus reducing the potential to be influenced by later incoming As and Pb. Therefore when As and Pb were introduced to soils 7 days later than Cd, they did not affect Cd partitioning into soils. To sum up, under this exposure scenario, loadings and time length As, Cd and Pb co-existed as well as time interval of spiking were important for potential interaction.

Combining findings of these two scenarios, it is therefore assumed additive effect of mixed contaminants (As, Cd and Pb) for health risk assessment purposes under the following five situations:

(1) As, Cd and Pb are aged independently;

(2) As, Cd and Pb are aged simultaneously but loadings of As, Cd and Pb do not exceed the sorption capacity of soils;

(3) As, Cd and Pb are aged together in soils which are alkaline;

(4) As, Cd and Pb enter the same soil sequentially at long time intervals (e.g. ≥7 days); and

(5) As, Cd and Pb are simultaneously aged for a long time (90 days in the current study).

This is the first study to elucidate the interactive effects of As, Cd and Pb on their respective bioaccessibility, of these elements which entered the soil and aged under various conditions. Contaminant concentrations, spiking manners (sequence of an element entering the soil) and ageing time are important factors needed to be considered when assessing bioaccessibility of mixed contaminants in soils. These results add new

knowledge to current risk assessment by improving the accuracy of predicting bioaccessibility of mixed contaminants.

7.1.4 Uptake of As, Cd and Pb in HepG2 cells as well as their interaction during uptake

HepG2 cell line (human hepatocyte) was selected as *in vitro* representative of liver, the key detoxifying organ, to probe the uptake of As, Cd and Pb into target organ cells after being solubilised in human digestive system as well as the interaction effects of As, Cd and Pb on their respective uptake (Chapter 5).

A linear relationship was gained between As dosing concentration and As uptake whilst Cd uptake can be described in a nonlinear regression model. The inflexion in the curve of Cd uptake probably resulted from the increased toxicity at high Cd dosing levels. Uptake values of As were much lower than those of Cd, which may be attributed to the competition between As and phosphate in the culture medium for transporters. Moreover, the uptake of UBM-extracted As and Cd was significantly greater than that of pure As and Cd. The possible reason might be that thiol groups (e.g. BSA, mucin, pepsin and their breakdown products) in dosing solution can bind As or Cd and thiol-bound metal/metalloid can be absorbed into cells via membrane carriers for amino acids, proteins or by endocytosis.

73-87% of UBM-extracted Pb precipitated within 0.5 hour when added to DMEM. Inorganic ions in DMEM (e.g. phosphate, sulphate and carbonate) which were in hundreds or thousands of mmol L^{-1}, could react with Pb to generate insoluble compounds during incubation time. Due to this limitation, uptake of Pb measured in most concentrations was either below the detection limit or in significant variations.

Interaction study indicates the accumulation of Cd in HepG2 cells was decreased by As or Pb whilst uptake of As was not affected by either Cd or Pb. These *in vitro* results pointed similar direction since *in vivo* studies showed mainly additive or less than additive effects among binary mixtures of As, Cd and Pb. Therefore, HepG2 cells maybe a useful cell model to represent *in vivo* liver tissue when investigating accumulation interaction of As and Cd on hepatic level. However, it is of limited value for Pb considering the precipitation problem. It is therefore suggested that speciation should be taken into consideration when dealing with *in vitro* system, especially for Pb which can form precipitates with a wide range of anion species.

The exploration of suitable *in vitro* model as a potential surrogate for *in vivo* system is in response to the global tendency of replacing, reducing and refining animal experiments (the so-called 3R principles in animal ethics). In this thesis, HepG2 cells demonstrate its potential for As and Cd studies, but without calibration with *in vivo* data, it can hardly gain wide acceptance. Soil samples which have been put through animal bioavailability model are highly recommended to go through the "bioaccessibility-uptake" system in the present study in order to validate and correlate with current results.

7.1.5 Effects of PAHs on As, Cd and Pb with respect to bioaccessibility and uptake in HepG2 cells

Naphthalene (NAP), phenanthrene (PHE), pyrene (PYR) and benzo[a]pyrene (B[a]P) were initially selected as representatives of the PAHs family to investigate their effects on the bioaccessibility of As, Cd and Pb as well as uptake in HepGe2 cells (Chapter 6).

Temporal change in soil PAH concentrations was first analysed. Results show that NAP was reduced to 2 mg kg^{-1} by 30 days, presumably because of its high volatility and rapid degradation rate. The declines in total concentrations of PYR and B[a]P (35-52%) were

less than that of PHE (58-65%) due to their respective higher hydrophobicity and resistance to microbial degradation with increased aromatic rings. Quantification methods for PAHs, sterilisation, spiking concentrations and procedures were key variables for the dissipation of PAHs with time. Based on the stability of concentrations in soils, PYR and B[a]P were chosen for interaction study with As, Cd and Pb.

Bioaccessibility interaction studies were carried out to mimic two exposure scenarios as described above. As, Cd, Pb and two PAHs (PYR, B[a]P) were either spiked in individual soils (independent ageing) or the same soils (simultaneous ageing). Results of Scenario one demonstrate that bioaccessibility of As, Cd and Pb was not affected by the co-existing PYR or B[a]P in the simulated digestive fluids (p>0.05). These results were in accordance to what was observed in Chapters 2 and 3, which indicates contaminants act independently with the functional groups of soil.

In the second exposure scenario, binary mixtures of As/Cd/Pb and PYR/B[a]P (As+PYR, As+B[a]P, Cd+PYR, Cd+B[a]P, Pb+PYR, Pb+B[a]P) were sequentially added to KBA or TAA soils. Bioaccessibility of As, Cd and Pb was analysed at 7, 30 and 90 days. During this period of ageing, the occurrence of PYR or B[a]P in soils did not show impact on the solubility of As/Cd/Pb in simulated digestive system (p>0.05). Previous studies have reported the influences of metal/metalloids on the behaviours of PAHs (e.g. sorption, partitioning, dissipation, bioaccessibility). However, no researcher has revealed the influence of PAHs on metal/metalloids in terms of bioaccessibility, which was also not detected in this study. Therefore, interaction between metal/metalloid and PAHs might not be reciprocal under some situations, e.g. in soil environment. It seems that PAHs in soils are more inclined to be influenced by metal/metalloid (e.g. affect microflora) than vice versa.

Effects of PYR and B[a]P on the uptake of As, Cd and Pb were tested by dosing HepG2 cells with UBM extraction solutions containing As/Cd/Pb and PYR/B[a]P. Results demonstrate that the uptake of As and Pb was not affected by PYR and B[a]P whilst the uptake of Cd was decreased by PYR and B[a]P, probably due to more damaged cell membrane. The reduction in Cd uptake at high concentrations (0.63/0.67 μmol L^{-1}) was more pronounced than that at low concentrations (0.28/0.29 μmol L^{-1}), which indicates interaction may be dose-dependent.

7.2 Conclusions

Arsenic, Cd and Pb reacted with soil components rapidly when spiked in soils and three-month ageing was sufficient for As/Cd/Pb to reach a steady bioaccessibility. The bioaccessibility in gastric phase was: Cd>Pb>As whilst in intestinal phase was As>Cd>Pb. Not only gastric bioaccessibility but also gastrointestinal bioaccessibility should be analysed during risk assessment. Gastric bioaccessibility is for the worst scenario, especially for highly soluble contaminants under acidic condition (e.g. Cd, Pb) whilst gastrointestinal bioaccessibility is more physiologically related. Laboratory spiked soils showed the reliability to represent field situations based on the bioaccessibility data obtained in this study. The United BARGE Method was proved to be a reliable and consistent *in vitro* bioaccessibility system.

Strong linear relationships, as expected, exist between bioaccessible As/Cd/Pb and total As/Cd/Pb in soils. Organic carbon, Fe oxide and Al oxide in soils were negatively related with bioaccessibility of As, Cd and Pb in the intestinal phase. Mineralogy (e.g. carbonate) seems to be a key controller for Pb bioaccessibility. Interpretation of soil-bioaccessibility

relationships based on values of r^2 (goodness of fit) should be taken with caution due to limited sample size. Inclusion of more soil types would help to avoid any incidental correlation between soil properties as well as to strengthen the conclusion of the current work.

Whether As, Cd and Pb could influence each other's bioaccessibility depend on several factors: (1) whether contaminants are aged independently in individual soils or simultaneously in same soils; (2) whether loadings of As, Cd and Pb exceed the sorption capacity of soils; (3) pH of soils; (4) the time intervals between each metal/metalloid enter the same soils; and (5) the length of time that multiple contaminants have been aged simultaneously.

In this current study, interaction was only observed in acidic soils with limited binding sites where As, Cd and Pb was spiked in the same soil sample at 24-hour interval: bioaccessibility of Cd in the intestinal phase was increased temporarily in the presence of Pb and As whilst Pb and As bioaccessibility was not affected. Under most environmental conditions, additive effects can be assumed when assessing bioaccessibility of mixed contaminants of As, Cd and Pb.

HepG2 cell line (human hepatocyte) maybe a promising cell device to investigate accumulation and interaction of As and Cd on hepatic level. Speciation should be taken consideration in *in vitro* cell culture system, especially for Pb.

No effects of PAHs (PYR and B[a]P) on bioaccessibility of As, Cd and Pb were noticed either in independently-aged or simultaneously-aged soils. However, interaction was observed during the uptake in HepG2 cells: the uptake of Cd was decreased by PYR/B[a]P whilst the uptake of As and Pb remained unchanged. It seems that the uptake of metal/metalloid at toxic doses, e.g. Cd here, was more easily affected (inhibition) as a mechanism of protection. Also, the difference in interaction results with regard to

bioaccessibility and uptake suggests that interaction patterns may vary with physiological processes and should be interpreted individually.

Results of bioaccessibility of mixed As, Cd and Pb (or mixed with PAHs) provide significant implications for clarifying situations where additive effects can be expected rather than assuming additivity in all situations, which is of great importance for risk assessment of mixtures.

7.3 Future work

(1) Spiked concentrations of As, Cd and Pb in soils were based on the Health Investigation Level A without measuring the sorption capacities of soils for As, Cd and Pb. In future study, sorption capacities for As, Cd and Pb should be measured in order to explore the interaction pattern when concentrations of As, Cd and Pb-spiked in soils exceed the soil capacity (over-loaded concentrations) and compare with the data obtained in this current study.

(2) As mentioned before, there was a strong correlation between contents of Fe oxide and Al oxide in seven types of soils reported in this thesis. Future work should include more soil types which have distinct contents of Fe oxide and Al oxide to further elucidate the role that Fe oxide and Al oxide individually play in bioaccessibility.

(3) Results of Pb bioaccessibility indicate the importance of soil mineralogy to Pb bioaccessibility. In this study, carbonate is a key soil mineral which leads to high gastric bioaccessibility and extremely low intestinal bioaccessibility. Therefore, it is prudent that more carbonate soils should be investigated in the future.

(4) It remains to be investigated that whether the manner by which contaminants are spiked could influence the interaction pattern among simultaneously-aged As, Cd and Pb in soils. Firstly, it is not clear that if the order by which As, Cd and Pb are introduced into soils could make a difference. Secondly, it is also not known that whether mixtures are added into soil as one solution or separate solutions can change the results. These questions should be addressed in the future work.

(5) Although UBM is physiological-based, it is an *in vitro* system which simulates the human digestive environment. *In vivo* experiments in the future would help to validate the interaction results gained in this study by dosing animals with independently-aged or simultaneously-aged contaminants in soils.

(6) Hep G2 cell lines shows the potential to be useful *in vitro* model to study the accumulation and interaction of As and Cd at hepatic level. However, endeavour should be made in the future to validate this cell model against animal model by putting soils which have bioavailability data of liver through the UBM-HepG2 system used in the current study.

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Appendices

Appendix 1: UBM methodology and fluid ingredients

Number of Figures: 1

Number of Tables: 1

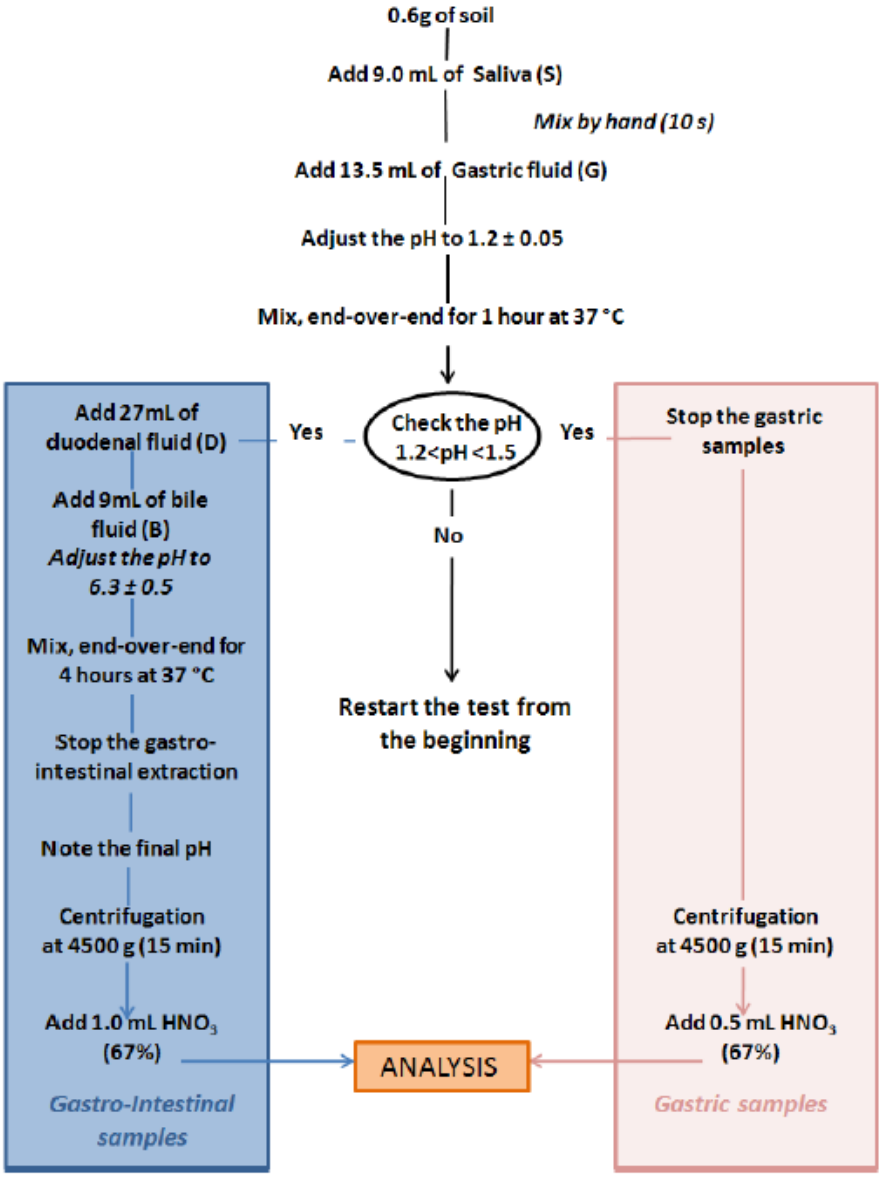


Figure S1 Schematic diagram of UBM methodology (available online: https://www.bgs.ac.uk/barge/docs/BARGE_UBM_DEC_2010.pdf)

Table S1 Composition of the digestive solutions used in UBM test

Saliva	Gastric phase	Intestinal phase	Bile
Inorganic solution (500 mL)			
KCl 896 mg	NaCl 2,752 mg	NaCl 7,012 mg	NaCl 5,259 mg
NaH_2PO_4 888 mg	NaH_2PO_4 266 mg	$NaHCO_3$ 5,607 mg	$NaHCO_3$ 5,785 mg
KSCN 200 mg	KCl 824 mg	KH_2PO_4 80 mg	KCl 376 mg 180
Na_2SO_4 570 mg	$CaCl_2$ 400 mg	KCl 564 mg	180 µL HCl 37%
NaCl 298 mg	NH_4Cl 306 mg	$MgCl_2$ 50 mg	
1.8 mL NaOH 1.0 M	8.3 mL HCl 37%	180 µL HCl 37%	
Organic solution (500 mL)			
Urea 200 mg	Glucose 650 mg	Urea 100 mg	Urea 250 mg
	Glucuronic acid 20 mg		
	Urea 85 mg		
	Glucosamine hydrochloride 330 mg		
Added compounds			
Amylase 145 mg	Bovine albumin 1 g	$CaCl_2$ 200 mg	$CaCl_2$ 222 mg
Mucin 50 mg	Mucin 3 g	Bovine albumin 1 g	Bovine albumin 1.8 g
Uric acid 15 mg	Pepsin 1 g	Pancreatin 3 g	Porcine bile 6 g
		Lipase 500 mg	

Appendix 2: Chapter 2 supplementary materials

Number of Figures: 3

Numbers of Tables: 2

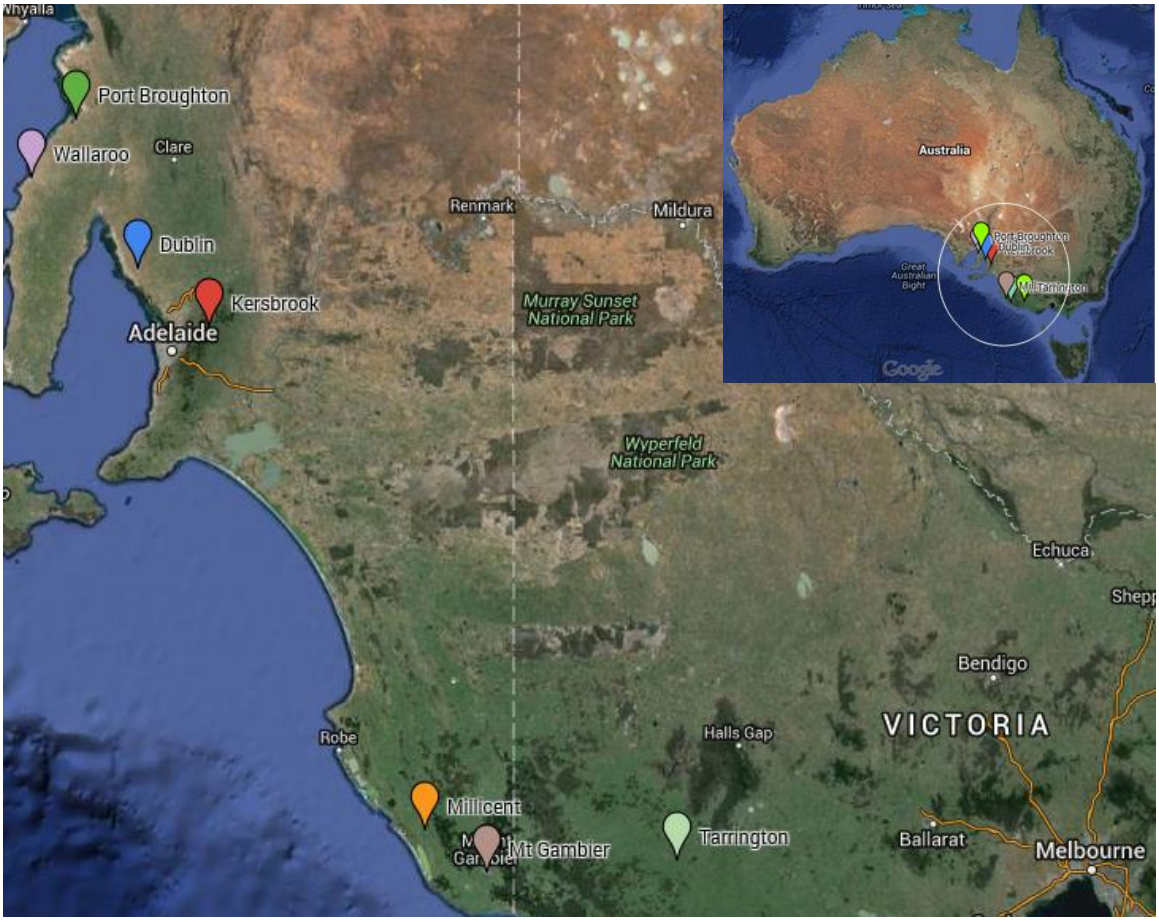
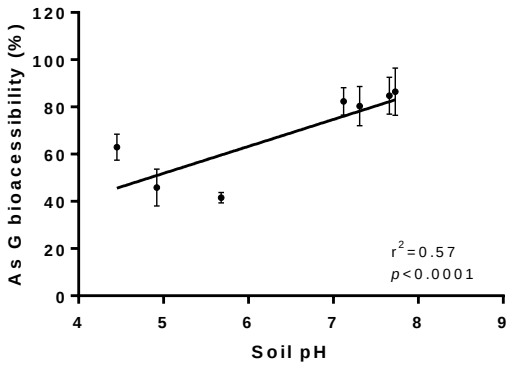


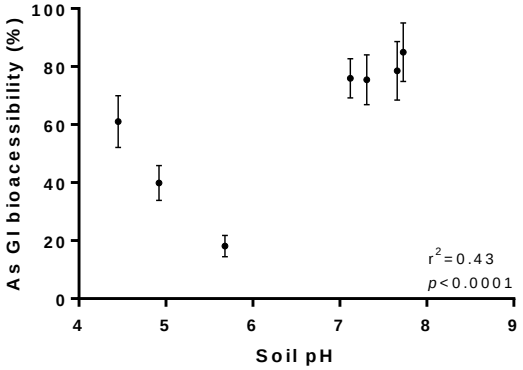
Figure S1 Locations of sampling sites in States of Victoria and South Australia, Australia (marked on Google Maps in relation to the capital cities of Melbourne and Adelaide respectively).

Table S1 Measured total As and Cd in the <250 μm fraction of spiked and control soils after ageing for one year

Soil code	MIA		MGA		KBA		TAA		WRA		PBA		DUA	
	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]	spike (mg kg^{-1})	analysis (mg kg^{-1})[a]
As	control	6.2	control	5.8	control	2.1	control	2.8	control	2.2	control	1.3	control	0.9
	100	69	100	128	100	80	100	76	100	80	100	86	100	101
	250	200	250	313	250	145	250	206	250	211	250	247	250	248
	1000	732			500	402	500	455	500	522				
					1000	1051	1000	903						
							2000	1930						
Cd	control	1.1	control	1.4	control	1.1	control	1.4	control	1.4	control	1.3	control	1.9
	25	20	50	102	25	26	25	30	50	50	50	41	50	40
	50	51	100	185	50	61	50	63	100	95	250	152	100	75
	250	174	250	380	100	91	100	110	250	258	500	338	250	218
	500	332	500	833	250	261	250	306	500	570			500	413
					500	671	500	775						

[a] Data represent the mean of duplicate analysis. Values varied by less than 5%. “Spike” means theoretical spiked concentration in soils; “Analysis” means measured concentrations in soils after soils have been aged and sieved <250 μm .


As G bioaccessibility (%)
Soil pH
r²=0.57
p<0.0001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Soil pH
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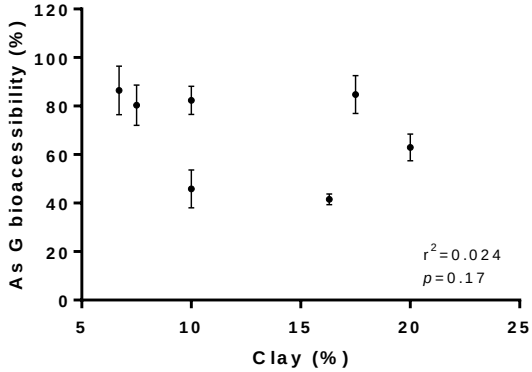
p<0.0001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Clay (%)
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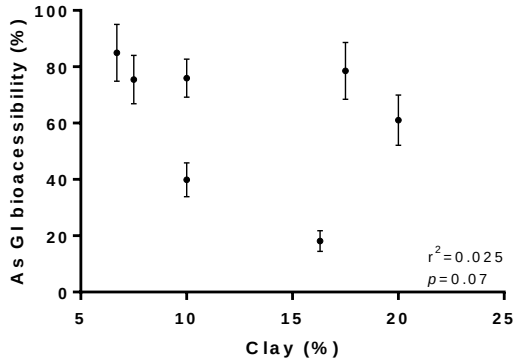
p=0.17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Clay (%)
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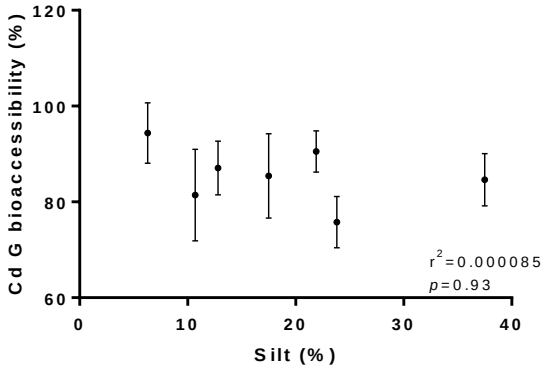
p=0.07


Cd G bioaccessibility (%)
Silt (%)
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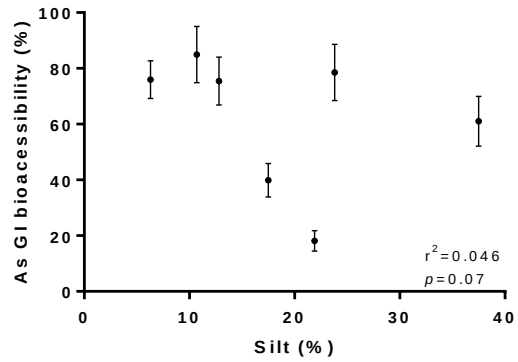
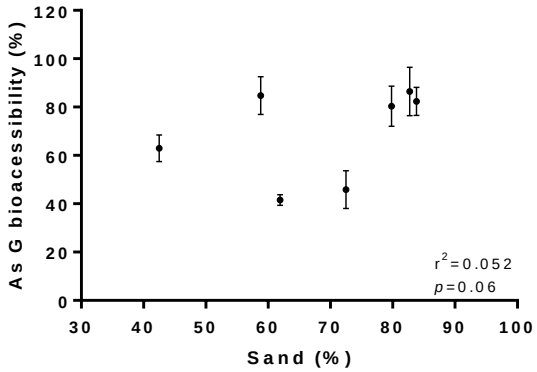
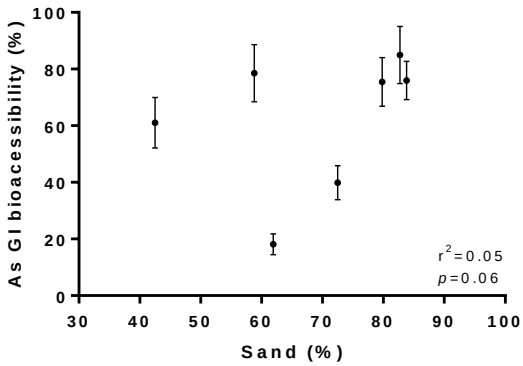
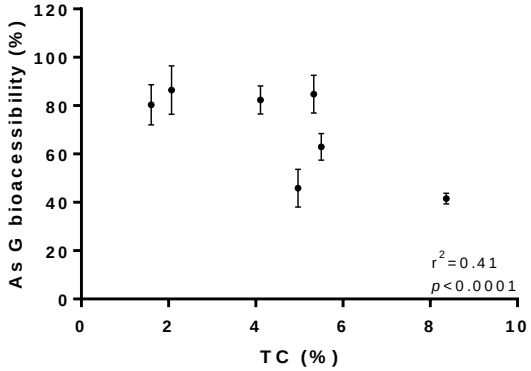
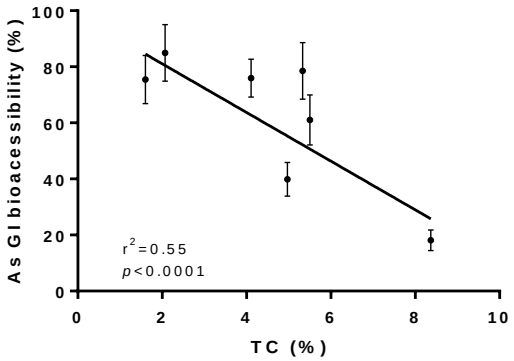
p=0.93

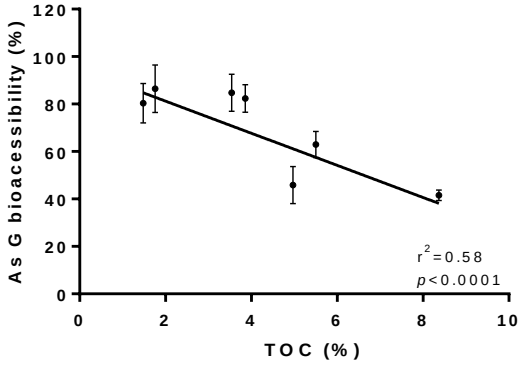

As GI bioaccessibility (%)
Silt (%)
r²=0.046
p=0.07

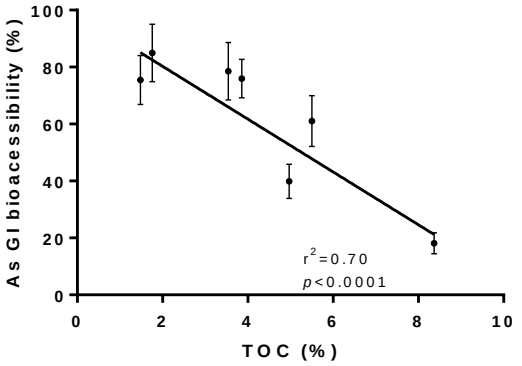

As G bioaccessibility (%)
Sand (%)
r²=0.052
p=0.06

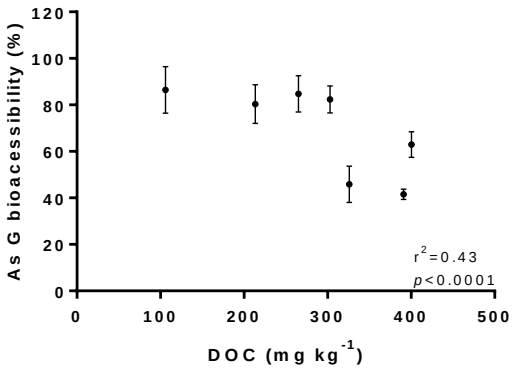

As GI bioaccessibility (%)
Sand (%)
r²=0.05
p=0.06

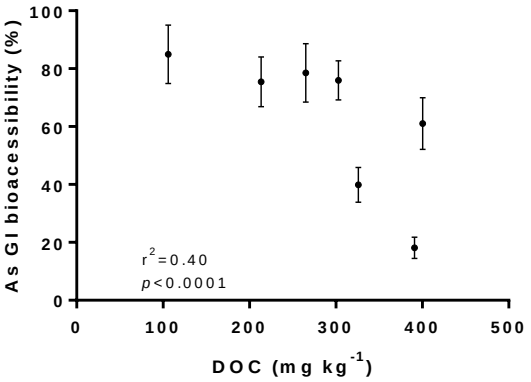

As G bioacessibility (%)
TC (%)
r²=0.41
p<0.0001

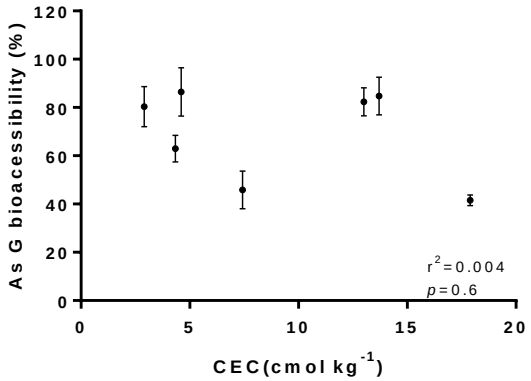

As GI bioacessibility (%)
TC (%)
r²=0.55
p<0.0001

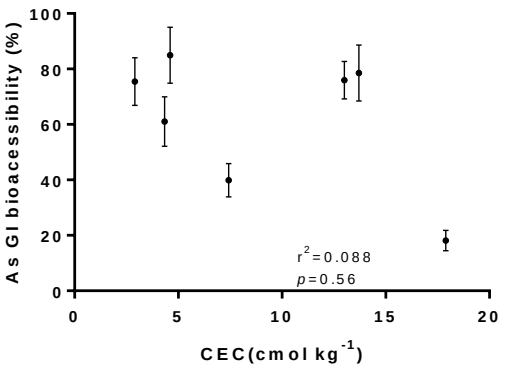

As G bioacessibility (%)
TOC (%)
r²=0.58
p<0.0001


As GI bioacessibility (%)
TOC (%)
r²=0.70
p<0.0001


As G bioacessibility (%)
DOC (mg kg-1)
r²=0.43
p<0.0001


As GI bioacessibility (%)
DOC (mg kg-1)
r²=0.40
p<0.0001


As G bioacessibility (%)
CEC(cmol kg-1)
r²=0.004
p=0.6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CEC(cmol kg-1)
r²=0.088
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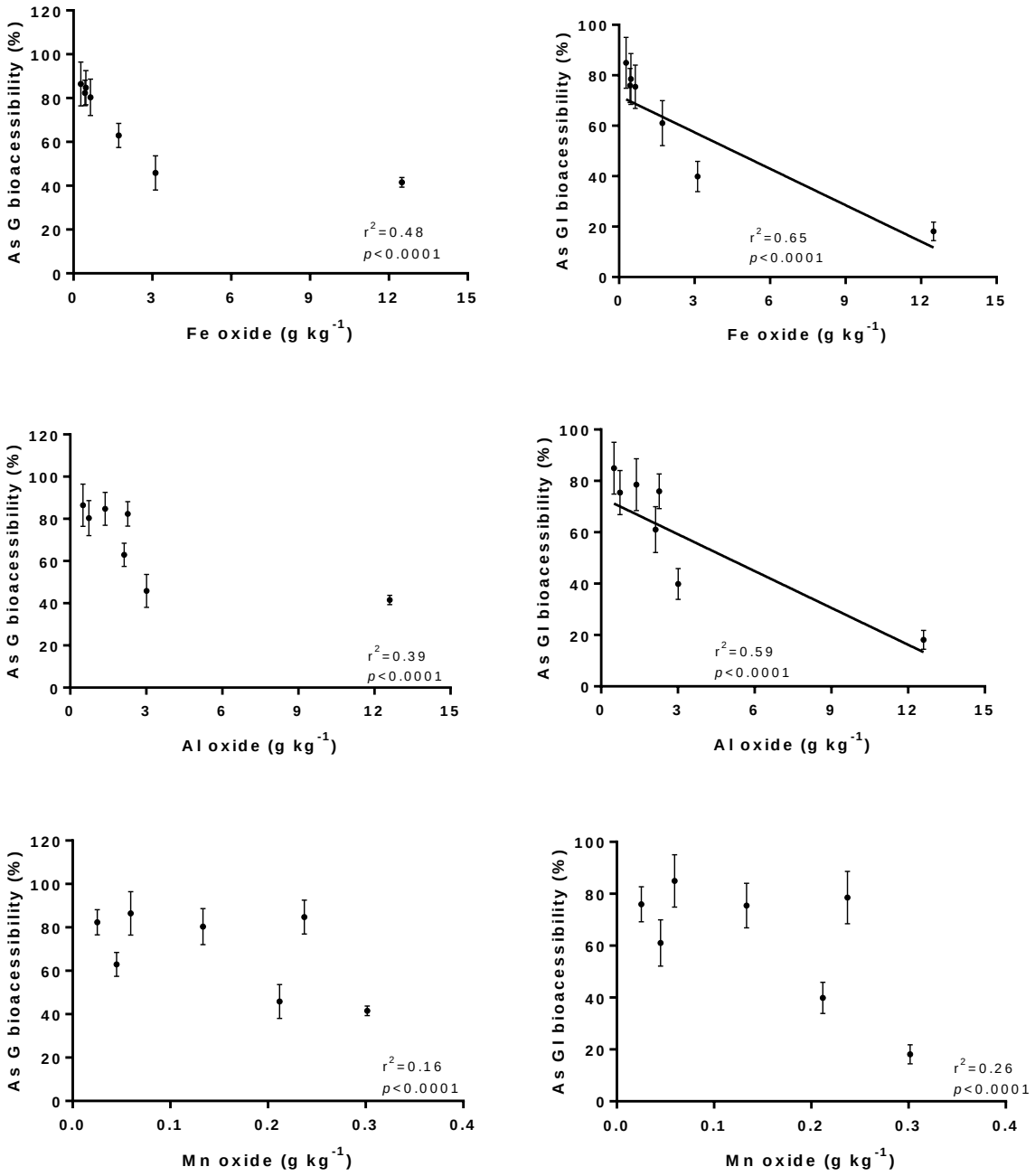
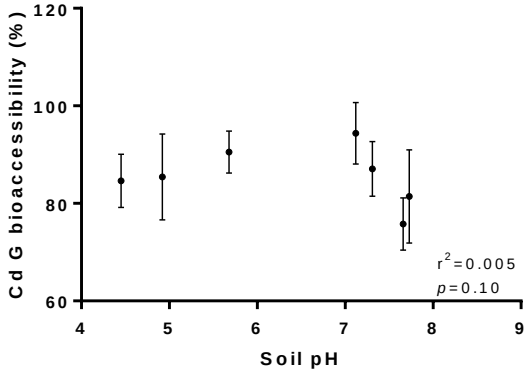
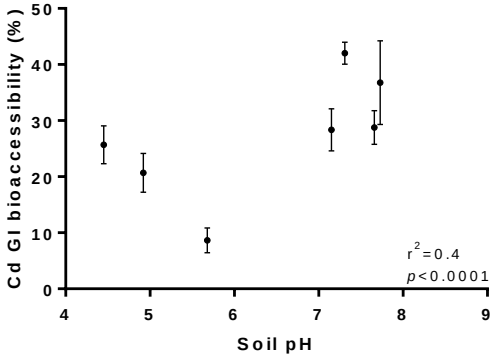
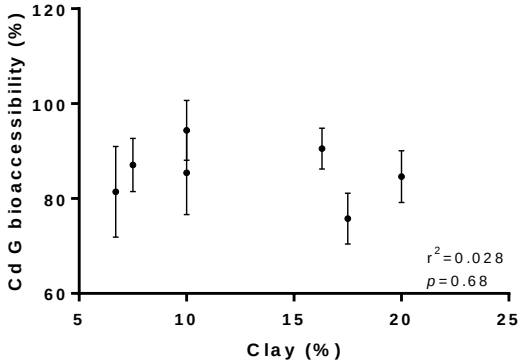
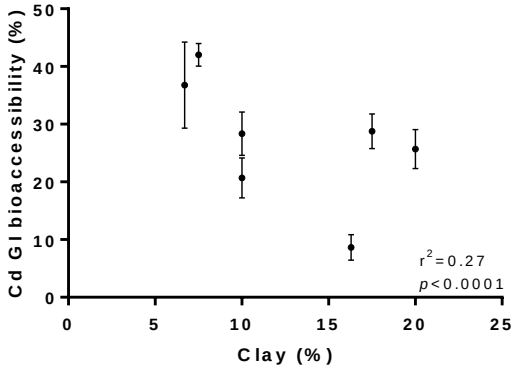


Figure S2 Linear regressions between As bioaccessibility and soil properties. Data represent mean ± standard variation. Figures with lines mean relationship is significant (r^2>0.5, *p*<0.0001). "G" means gastric phase and "GI" means intestinal phase.


Cd G bioaccessibility (%)
120
100
80
60
4
5
6
7
8
9
Soil pH
r2=0.005
p=0.10


Cd GI bioaccessibility (%)
50
40
30
20
10
0
4
5
6
7
8
9
Soil pH
r2=0.4
p<0.0001


Cd G bioaccessibility (%)
120
100
80
60
5
10
15
20
25
Clay (%)
r2=0.028
p=0.68


Cd GI bioaccessibility (%)
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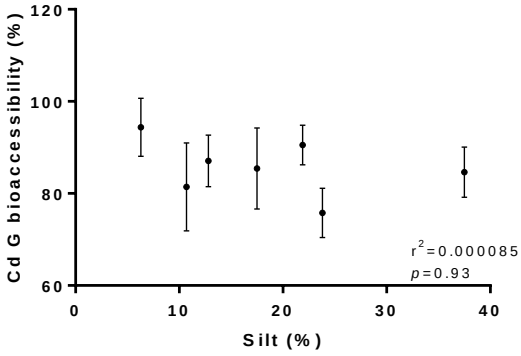
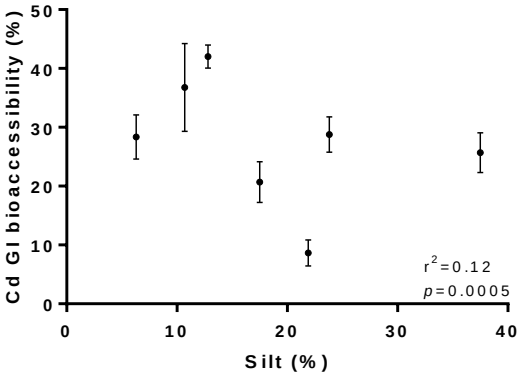
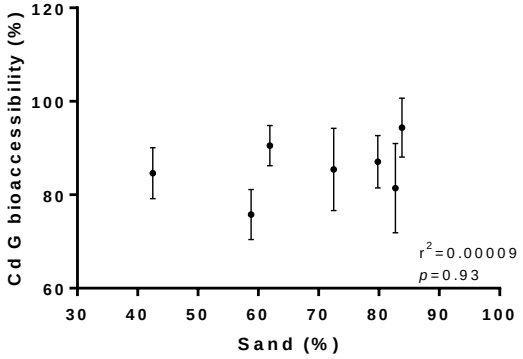
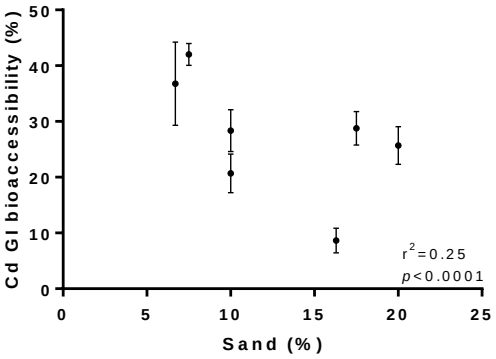
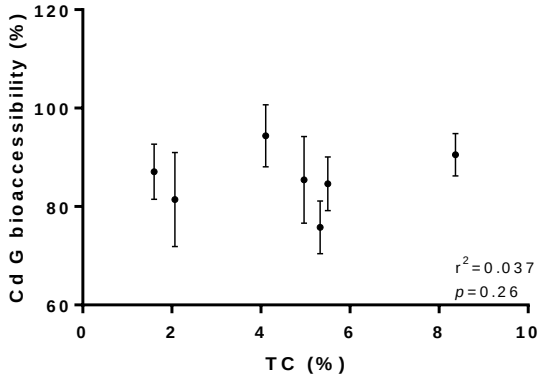
40
30
20
10
0
0
5
10
15
20
25
Clay (%)
r2=0.27
p<0.0001

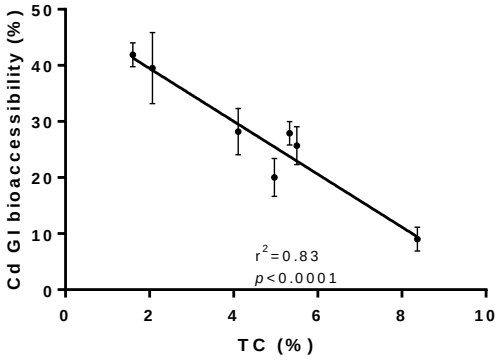

Cd G bioaccessibility (%)
120
100
80
60
0
10
20
30
40
Silt (%)
r2=0.000085
p=0.93

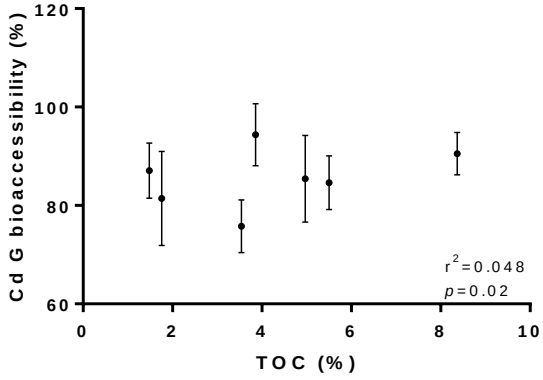

Cd GI bioaccessibility (%)
50
40
30
20
10
0
0
10
20
30
40
Silt (%)
r2=0.12
p=0.0005

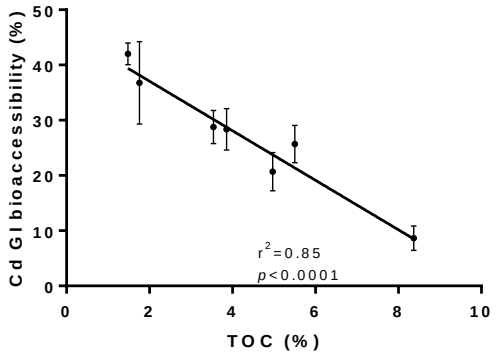

Cd G bioaccessibility (%)
120
100
80
60
30
40
50
60
70
80
90
100
Sand (%)
r2=0.00009
p=0.93

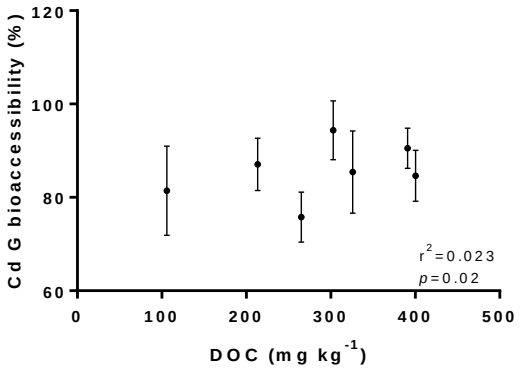

Cd GI bioaccessibility (%)
50
40
30
20
10
0
0
5
10
15
20
25
Sand (%)
r2=0.25
p<0.0001

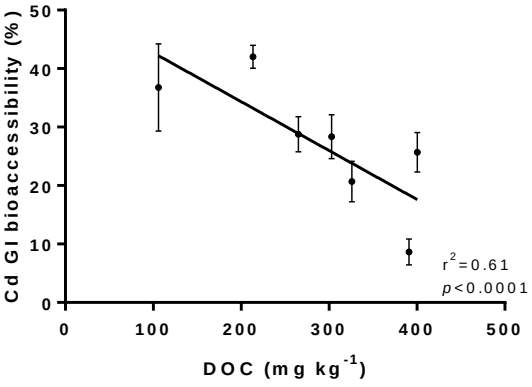

Cd G bioaccessibility (%)
120
100
80
60
0
2
4
6
8
10
TC (%)
r²=0.037
p=0.26

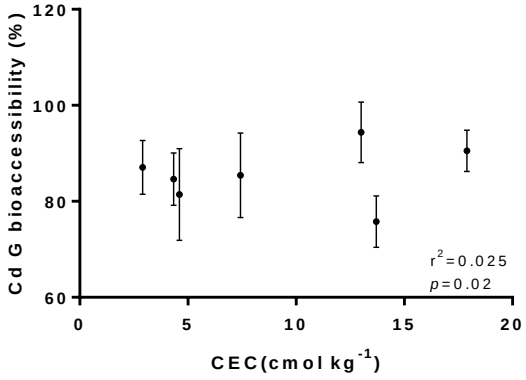

Cd GI bioaccessibility (%)
50
40
30
20
10
0
0
2
4
6
8
10
TC (%)
r²=0.83
p<0.0001

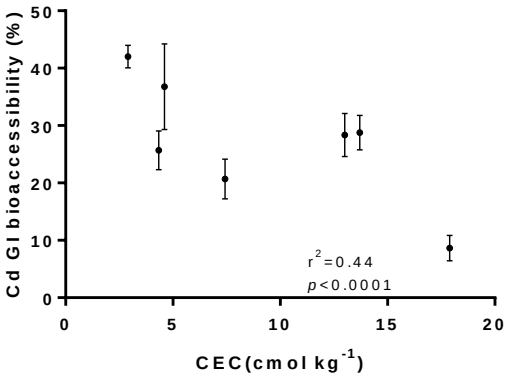

Cd G bioaccessibility (%)
120
100
80
60
0
2
4
6
8
10
TOC (%)
r²=0.048
p=0.02


Cd GI bioaccessibility (%)
50
40
30
20
10
0
0
2
4
6
8
10
TOC (%)
r²=0.85
p<0.0001


Cd G bioaccessibility (%)
120
100
80
60
0
100
200
300
400
500
DOC (mg kg-1)
r²=0.023
p=0.02


Cd GI bioaccessibility (%)
50
40
30
20
10
0
0
100
200
300
400
500
DOC (mg kg-1)
r²=0.61
p<0.0001


Cd G bioaccessibility (%)
120
100
80
60
0
5
10
15
20
CEC(cmol kg-1)
r²=0.025
p=0.02


Cd GI bioaccessibility (%)
50
40
30
20
10
0
0
5
10
15
20
CEC(cmol kg-1)
r²=0.44
p<0.0001

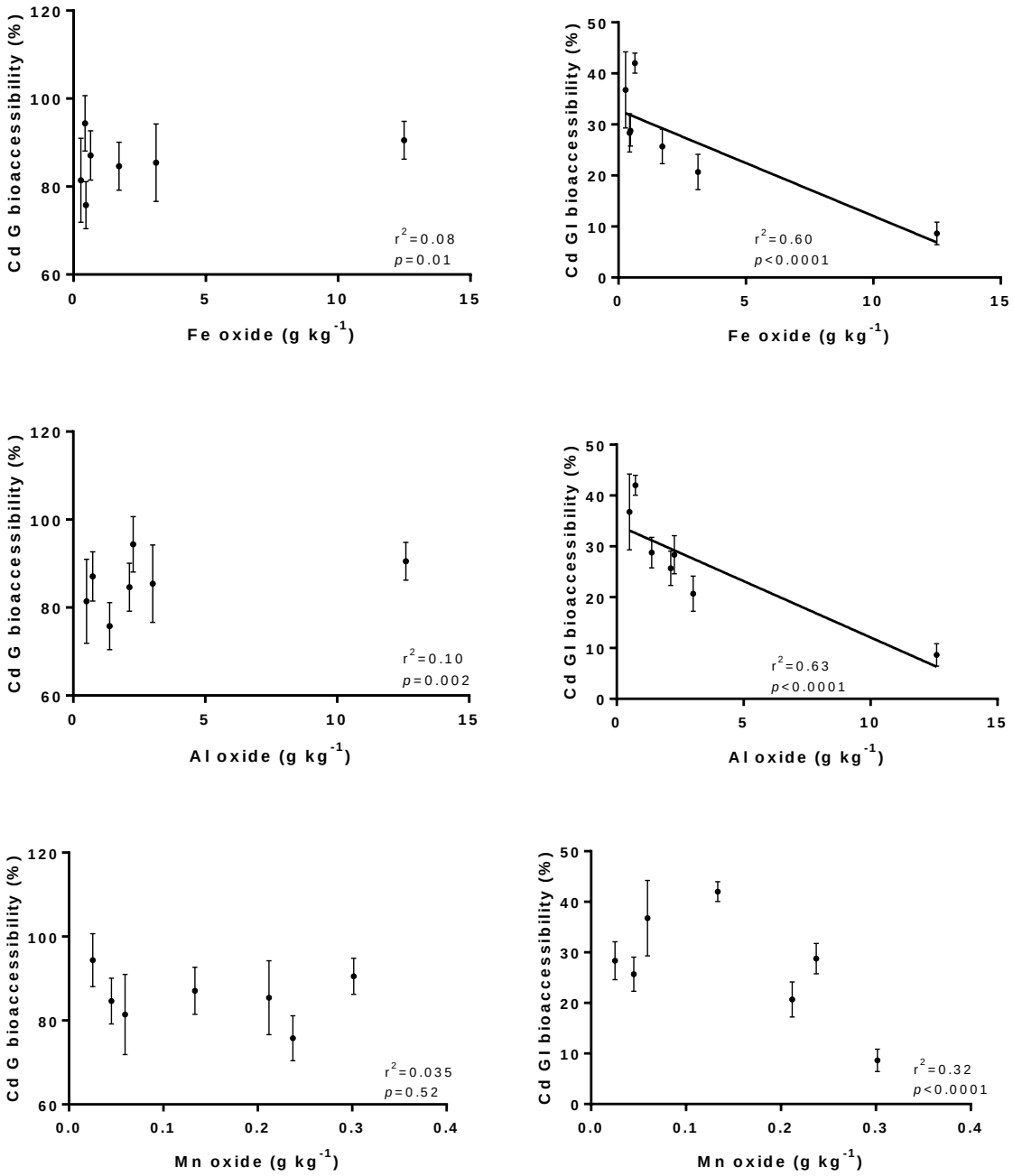


Figure S3 Linear regressions between Cd bioaccessibility and soil properties. Data represent mean ± standard variation. Figures with lines mean relationship is significant (r^2>0.5, *p*<0.0001). "G" means gastric phase and "GI" means intestinal phase.

Table S2 p values of Students' t-test

Soil code	As(Cd) bioaccessibility			Cd(As) bioaccessibility		
	Total As (mg kg^{-1})	Gastric bioaccessibility	Intestinal bioaccessibility	Total Cd (mg kg^{-1})	Gastric bioaccessibility	Intestinal bioaccessibility
MIA	69 (51)	0.17	0.27	51 (69)	0.41	0.54
	732 (332)	0.13	0.78	332 (732)	0.85	0.81
MGA	128 (102)	0.06	0.07	102 (128)	0.09	0.47
	313 (833)	0.07	0.11	833 (313)	0.28	0.07
KBA	80 (61)	0.07	0.25	61 (80)	0.7	0.22
	1051 (671)	0.85	0.19	671 (1051)	0.05	0.06
TAA	76 (63)	0.06	0.11	63(76)	0.29	0.24
	903 (775)	0.17	0.89	775 (903)	0.07	0.06
WRA	80 (50)	0.06	0.1	95 (80)	0.5	0.44
	522 (570)	0.6	0.47	570 (522)	0.25	0.14
PBA	86 (41)	0.19	0.08	41 (86)	0.88	0.69
	247(338)	0.45	0.45	338 (248)	0.92	0.58
DUA	101 (40)	0.51	0.79	40 (101)	0.18	0.25
	248 (413)	0.26	0.21	413 (248)	0.98	0.19

"As(Cd) bioaccessibility", 69(51) means 0.15 g soil spiked with 69 mg kg^{-1} As was mixed with 0.15 g soil spiked with 51 mg kg^{-1} Cd, etc; Bioaccessibility of As was compared with that of As in presence of Cd.

"Cd(Pb) bioaccessibility", 51(69) means 0.15 g soil spiked with 51 mg kg^{-1} Cd was mixed with 0.15 g soil spiked with 69 mg kg^{-1} As, etc; Bioaccessibility of Cd was compared with that of Cd in presence of As.

Appendix 3: Chapter 3 supplementary materials

Number of Tables: 5

Table S1 Total, bioaccessible concentrations and bioaccessibility of As, Cd and Pb in SRM 2710a and 2711a

SRM	metals/ metalloid	Total concentration			Bioaccessible metals/metalloid (mg kg^{-1})		Bioaccessibility metals/metalloid (%)	
		Certified value(mg kg^{-1})	Measured value(mg kg^{-1})	Recovery (%)	Gastric phase	Intestinal phase	Gastric phase	Intestinal phase
2710a	As	1540±10	1418 ± 105	92 ± 6.8	487±28	412±30	34.3±2.0	29.1±2.1
	Cd	12.3±0.3	10.7 ± 0.8	87 ± 6.1	4.8±0.3	2.8±0.33	44.9±2.8[a]	26.2±3.1[a]
	Pb	5520±30	5479 ± 84	99 ± 1.5	2817±313	407±78	51.4±5.7	7.4±1.4
2711a	As	107±5	90.5 ± 8.5	85 ± 7.9	46.5±2.7[b]	41.4±5.0[b]	51.4±3.0[c]	45.8±5.5[c]
	Cd	54.1±0.5	48.5 ± 4.1	85 ± 7.6	41.6±3.2[b]	13.8±0.7[b]	85.8±6.6	28.5±1.4
	Pb	1400±10	1321± 19	94 ± 1.4	1041 ±124[b]	50.4 ±4.6[b]	84.6±10.1	3.8±0.3

[a, b. c] means data were in agreement with the following literature [a](Roussel et al., 2010), [b](Wragg et al., 2011), [c](Li et al., 2015b)

Data represents the results of six replicates. Please note that there are plenty of published bioaccessibility data for SRM 2710 and 2711 but since SRM 2710a and 2711a are renewal materials of SRM 2710 and 2711, limited published data are available for comparison. Especially for SRM 2710a, it was sampled from a different location from 2710.

Table S2 Measured total As, Cd and Pb in the <250 µm fraction of spiked and control soils after ageing for one year compared with intended spiked concentration in soils <2 mm.

	MIA		MGA		KBA		TAA		WRA		PBA		DUA	
	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]	Spike (mg kg^{-1})	Analysis (mg kg^{-1})[a]
As[b]	control	6.2	control	5.8	control	2.1	control	2.8	control	2.2	control	1.3	control	0.9
	100	69	100	128	100	80	100	76	100	80	100	86	100	101
	250	200	250	313	250	145	250	206	250	211	250	247	250	248
	1000	732			500	402	500	455	500	522				
					1000	1051	1000	903						
							2000	1930						
Cd[b]	control	1.1	control	1.4	control	1.1	control	1.4	control	1.4	control	1.3	control	1.9
	25	20	50	102	25	26	25	30	50	50	50	41	50	40
	50	51	100	185	50	61	50	63	100	95	250	152	100	75
	250	174	250	380	100	91	100	110	250	258	500	338	250	218
	500	332	500	833	250	261	250	306	500	570			500	413
					500	671	500	775						
Pb	control	11	control	49	control	43	control	34	control	13	control	14	control	12
	500	680	250	453	400	569	250	309	250	289	400	445	400	443
	1000	1047	1139	1139	1200	1189	500	756	1000	1049	800	864	800	907
	4000	3367	2000	2636	2400	2224	1000	1436	2000	2219	2400	2332	2400	2495
	8000	5949	4000	7028	5000	4544	4000	3997	4000	3898	5000	3971	5000	4361
			8000	12225			8000	9345						

[a] Data represent the mean of duplicate analysis. Values varied by less than 5%. "Spike" means theoretical concentration that spiked in soils which were dried and sieved <2 mm; "Analysis" means measured concentrations in soils after soils have been aged and sieved <250 µm (UBM required the <250 µm particle size fraction of soils).[b] Data were published in XIA, et al., 2016.

Table S3 Total Ca in seven types of soils

Soil code	MIA	MGA	KBA	TAA	WRA	PBA	DUA
Total Ca (g kg^{-1}) [a]	11.24	9.18	1.45	2.45	49.48	13.58	2.34

[a] Data represent the mean of six replicates. Values varied by less than 5%.

Table S4 The effect of Pb on the bioaccessibility of As and Cd during UBM extraction.

	As(Pb) bioaccessibility			Cd(Pb) bioaccessibility		
Soil code	Total As (mg kg^{-1})[a]	Gastric bioaccessibility (%)[b]	Intestinal bioaccessibility (%)[b]	Total Cd (mg kg^{-1})[a]	Gastric bioaccessibility (%)[b]	Intestinal bioaccessibility (%)[b]
MIA	69	84±1.5	83±2.9	51	93±6.8	23±1.3
	69 (680)	79±1.1	74±1.1	51 (680)	96±4.6	25±2.5
	732	85±0.2	74±6.2	332	80±8.2	31±1.4
	732 (5949)	80±3.9	72±0.6	332 (3367)	92±5.5	31±3.2
MGA	128	41±1.9	17±2.4	38	86±1.9	6.3±0.4
	128 (453)	37±1.7	21±1.5	38 (453)	88±2.2	8±0.8
	313	43±2.7	18±6.5	833	94±3.8	12±0.3
	313 (7028)	37±1.1	19±1.4	833 (7028)	95±0.7	11±0.3
KBA	80	68±3.4	68±2.8	61	74±1.6	20±4.5
	80 (569)	66±5.8	64±2.1	61 (569)	76±4.9	15±1.6
	1051	68±1.5	60±11	671	88±1.5	28±4.4
	1051 (4544)	71±1.2	71±1.9	4544 (1051)	82±2.6	24±0.8
TAA	76	43±0.8	43±0.9	63	80±2.9	17±1.9
	76 (309)	41±1.3	39±1.9	63 (309)	80±3.0	15±0.7
	903	55±4.5	47±11	775	90±0.5	25±0.6
	903 (3997)	55±1..7	46±4.2	775 (3997)	97±4.0	23±1.5
WRA	80	94±0.5	89±0.6	50	94±5.9	31±4.4
	80 (289)	83±6.8	84±4.4	50 (289)	96±4.8	31±3.8
	522	76±2.3	69±2.2	570	82±8.9	33±5.4
	522 (3898)	69±2.9	67±1.1	570 (3898)	91±3.7	34±2.2
PBA	86	95±1.3	96±0.8	41	72±4.3	30±2.0
	85 (445)	91±2.6	94±4.0	41 (445)	76±3.2	31±6.1
	247	81±4.2	80±3.7	338	88±2.1	45±2.7
	247 (3971)	79±3.7	83±3.3	338 (3971)	85±5.5	39±3.9
DUA	101	83±9.3	73±11.2	75	90±5.7	41±0.6
	101 (443)	83±6.5	79±1.2	75 (443)	97±3.2	36±3.5
	248	79±5.5	80±2.8	413	85±12	44±3.1
	248 (4361)	81±2.8	81±1.0	413 (4361)	94±4.7	45±2.6

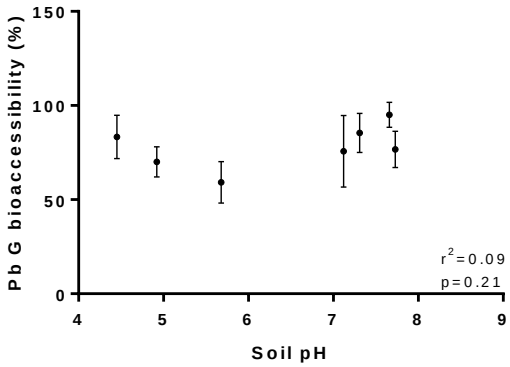
"As (Pb) bioaccessibility", 69(680) means 0.15 g soil spiked with 69 mg kg^{-1} As was mixed with 0.15 g soil spiked with 680 mg kg^{-1} Pb, etc; "Cd(Pb) bioaccessibility", 51(680) means 0.15 g soil spiked with 51 mg kg^{-1} Cd was mixed with 0.15 g soil spiked with 680 mg kg^{-1} Pb, etc.

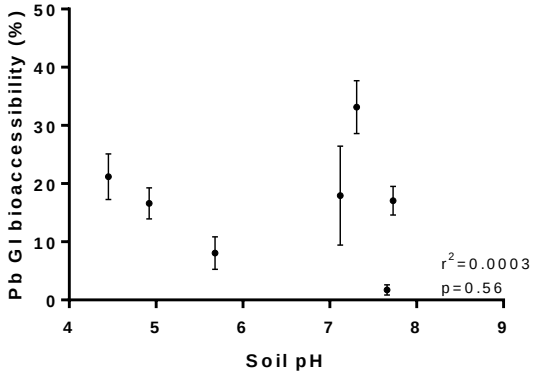
[a] Data represent the mean of measured concentrations (same as shown in Table S2, Appendix 3).

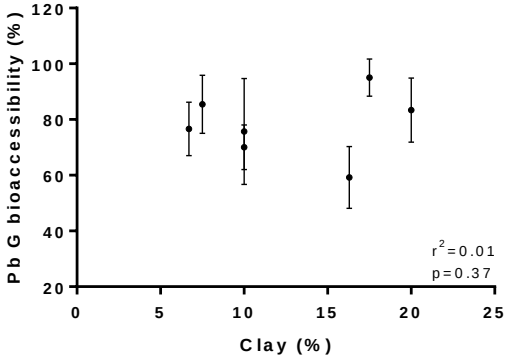
[b] Data represent the mean of at least triplicate measurement ± standard deviation (SD).

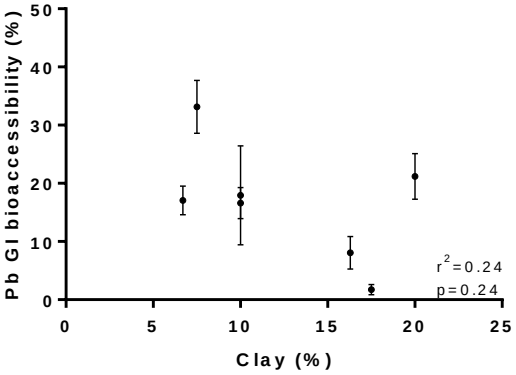
The <250 μm particle size was used for all measurements.

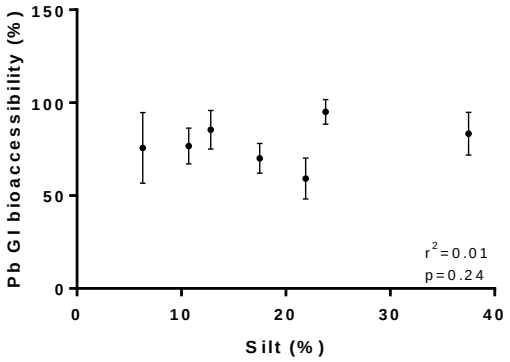
No significant differences (p>0.05) were detected between bioaccessibility of As and As (Pb) as well as between Cd and Cd (Pb).

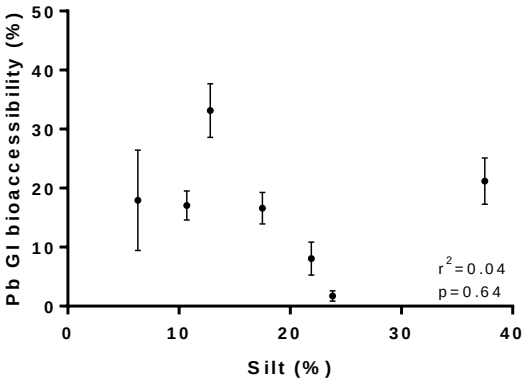

Pb G bioaccessibility (%)
150
100
50
0
4
5
6
7
8
9
Soil pH
r2=0.09
p=0.21

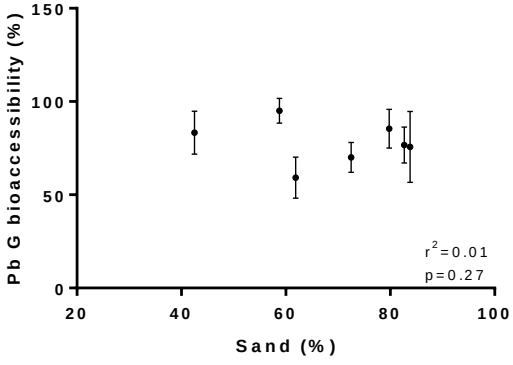

Pb GI bioaccessibility (%)
50
40
30
20
10
0
4
5
6
7
8
9
Soil pH
r2=0.0003
p=0.56

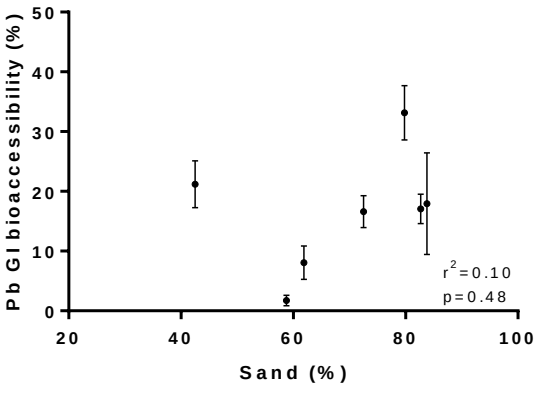

Pb G bioaccessibility (%)
120
100
80
60
40
20
0
5
10
15
20
25
Clay (%)
r2=0.01
p=0.37

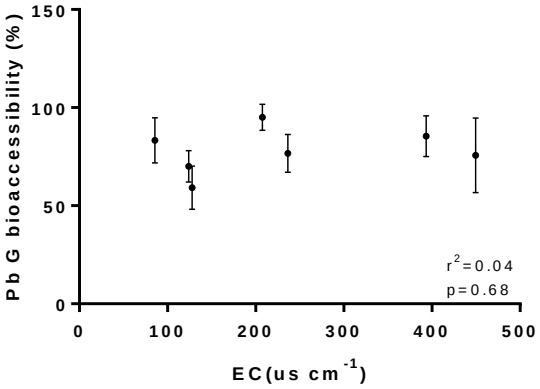

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
5
10
15
20
25
Clay (%)
r2=0.24
p=0.24

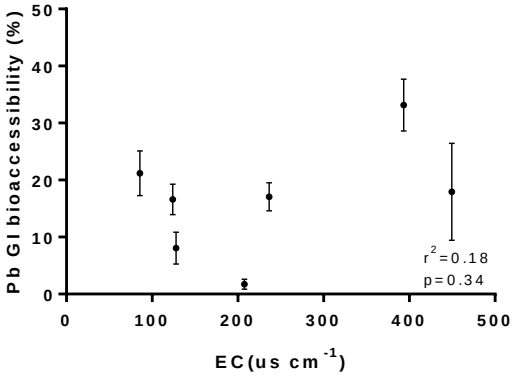

Pb GI bioaccessibility (%)
150
100
50
0
0
10
20
30
40
Silt (%)
r2=0.01
p=0.24

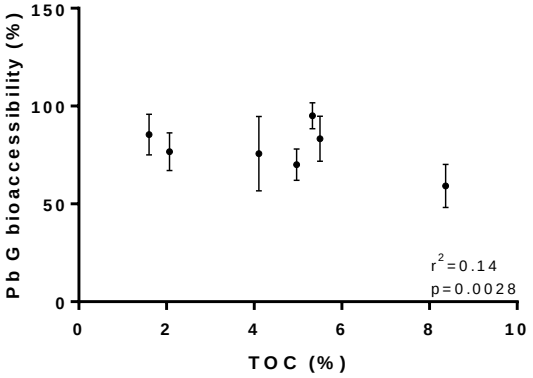

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
10
20
30
40
Silt (%)
r2=0.04
p=0.64

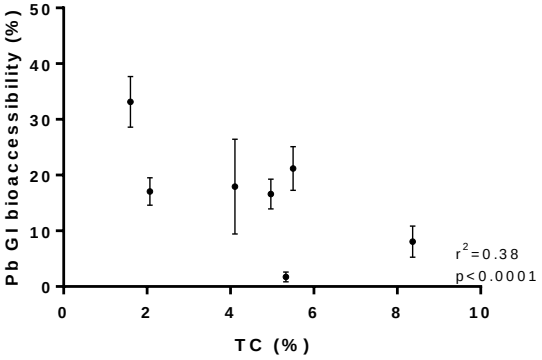

Pb G bioaccessibility (%)
150
100
50
0
20
40
60
80
100
Sand (%)
r2=0.01
p=0.27

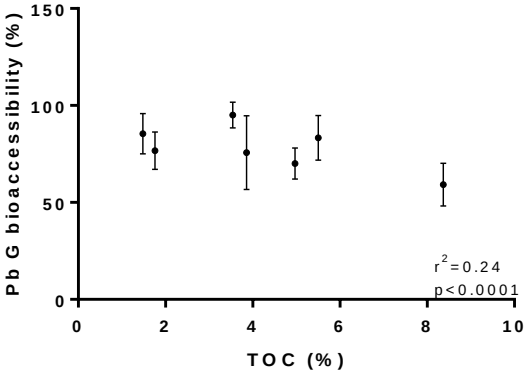

Pb GI bioaccessibility (%)
50
40
30
20
10
0
20
40
60
80
100
Sand (%)
r2=0.10
p=0.48

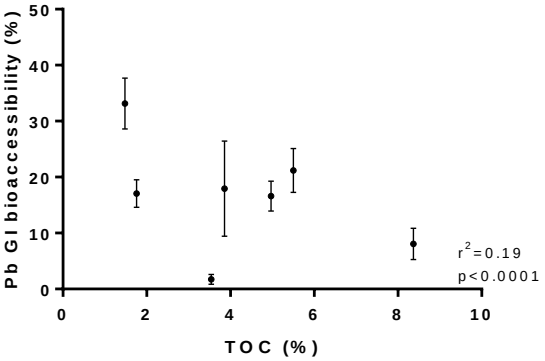

Pb G bioaccessibility (%)
150
100
50
0
0
100
200
300
400
500
EC(us cm-1)
r2=0.04
p=0.68

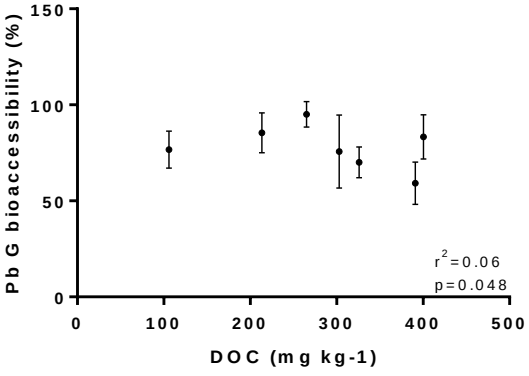

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
100
200
300
400
500
EC(us cm-1)
r2=0.18
p=0.34

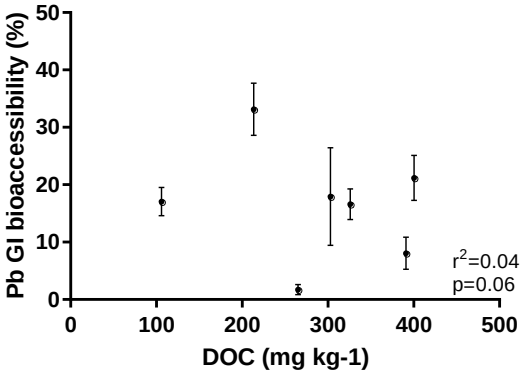

Pb G bioaccessibility (%)
150
100
50
0
0
2
4
6
8
10
TOC (%)
r2=0.14
p=0.0028

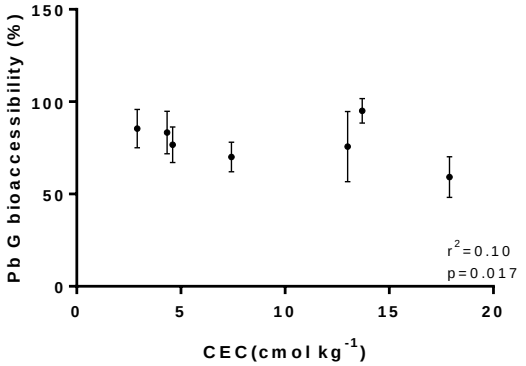

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
2
4
6
8
10
TC (%)
r2=0.38
p<0.0001

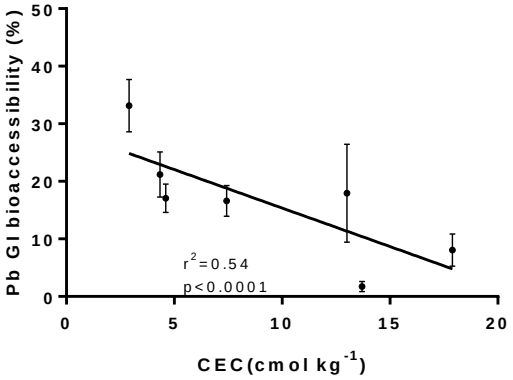

Pb G bioaccessibility (%)
150
100
50
0
0
2
4
6
8
10
TOC (%)
r2=0.24
p<0.0001

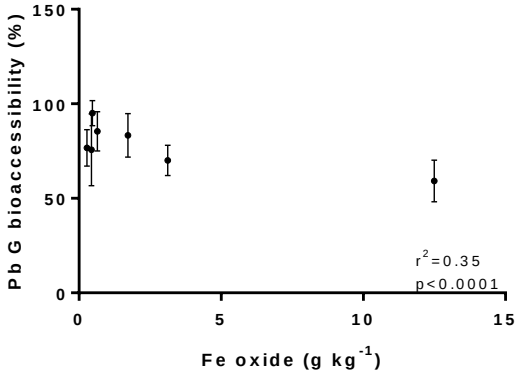

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
2
4
6
8
10
TOC (%)
r2=0.19
p<0.0001

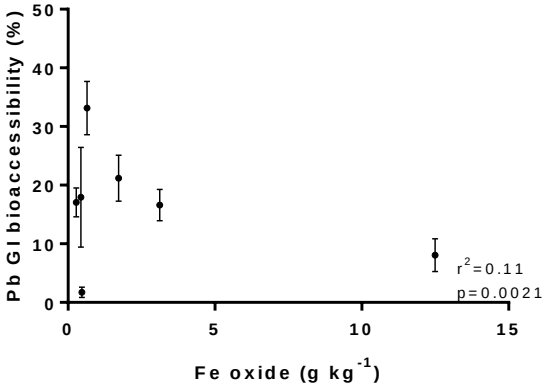

Pb G bioaccessibility (%)
150
100
50
0
0
100
200
300
400
500
DOC (mg kg-1)
r2=0.06
p=0.048

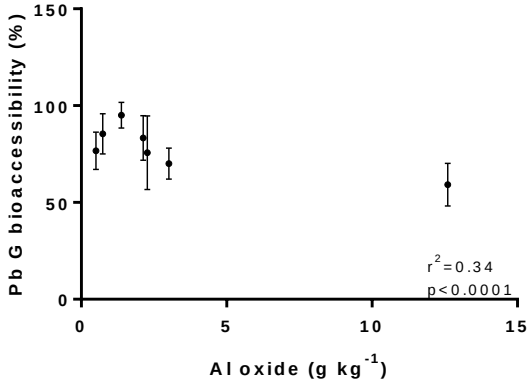

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
100
200
300
400
500
DOC (mg kg-1)
r2=0.04
p=0.06

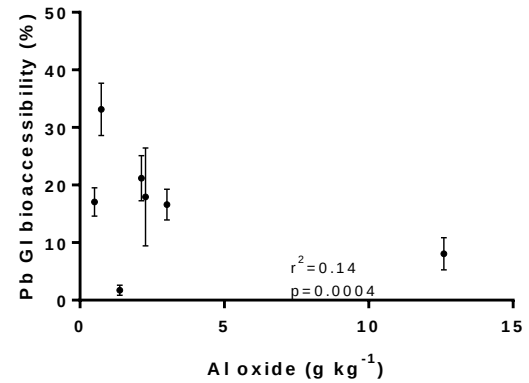

Pb G bioaccessibility (%)
150
100
50
0
0
5
10
15
20
CEC(cmol kg-1)
r2=0.10
p=0.017

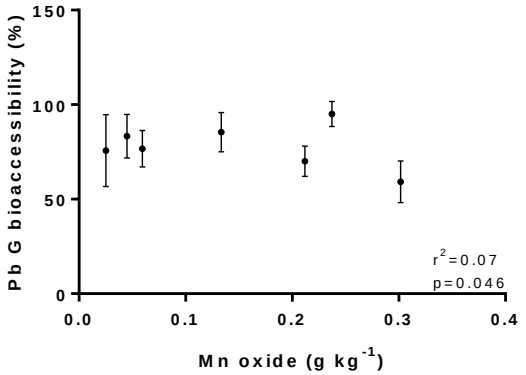

Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
5
10
15
20
CEC(cmol kg-1)
r2=0.54
p<0.0001

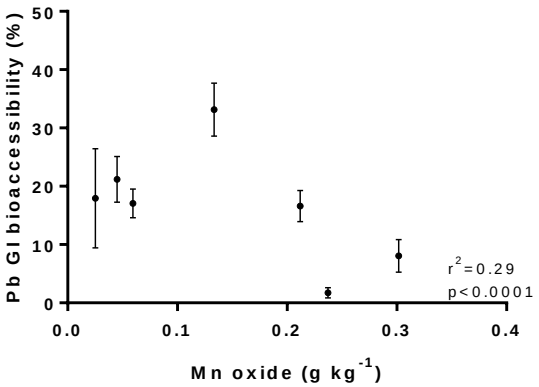

Pb G bioaccessibility (%)
150
100
50
0
0
5
10
15
Fe oxide (g kg-1)
r2=0.35
p<0.0001


Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
5
10
15
Fe oxide (g kg-1)
r2=0.11
p=0.0021


Pb G bioaccessibility (%)
150
100
50
0
0
5
10
15
Al oxide (g kg-1)
r2=0.34
p<0.0001


Pb GI bioaccessibility (%)
50
40
30
20
10
0
0
5
10
15
Al oxide (g kg-1)
r2=0.14
p=0.0004


Pb G bioaccessibility (%)
150
100
50
0
0.0
0.1
0.2
0.3
0.4
Mn oxide (g kg-1)
r2=0.07
p=0.046


Pb GI bioaccessibility (%)
50
40
30
20
10
0
0.0
0.1
0.2
0.3
0.4
Mn oxide (g kg-1)
r2=0.29
p<0.0001

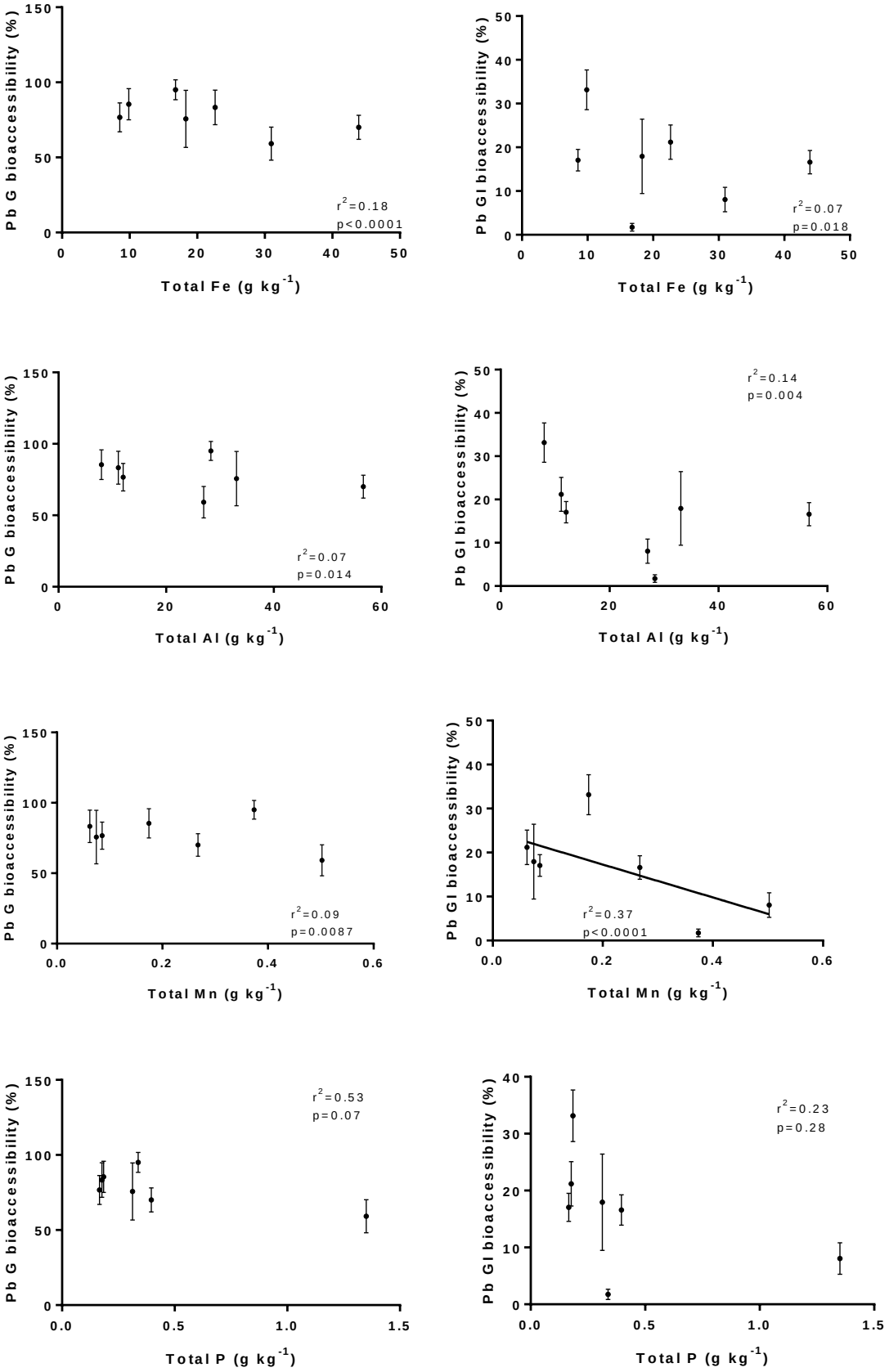


Figure S1 Linear regressions between Pb bioaccessibility and soil properties. Data represent mean ± standard variation. Figures with lines mean relationship is significant (r^2>0.5, *p*<0.0001). "G" means gastric phase and "GI" means intestinal phase.

Table S5 p values of Students' t-test

Soil code	Pb(As) bioaccessibility			Pb(Cd) bioaccessibility			As(Pb) bioaccessibility			Cd(Pb) bioaccessibility		
	Total Pb (mg kg^{-1})	Gastric bioaccessibility	Intestinal bioaccessibility	Total Pb (mg kg^{-1})	Gastric bioaccessibility (%)[b]	Intestinal bioaccessibility	Total As (mg kg^{-1})	Gastric bioaccessibility	Intestinal bioaccessibility	Total Cd (mg kg^{-1})	Gastric bioaccessibility	Intestinal bioaccessibility
MIA	680 (69)	0.94	0.43	680 (51)	0.09	0.78	69 (680)	0.06	0.06	51 (680)	0.23	0.36
	5949 (732)	0.08	0.17	3367 (332)	0.88	0.92	732 (5949)	0.11	0.98	332 (3367)	0.32	0.87
MGA	453 (128)	0.33	0.7	453 (38)	0.07	0.98	128 (453)	0.1	0.14	38 (453)	0.33	0.07
	7028 (313)	0.69	0.86	7028 (833)	0.08	0.31	313 (7028)	0.17	0.79	833 (7028)	0.81	0.14
KBA	569 (80)	0.33	0.09	569 (61)	0.1	0.28	80 (569)	0.46	0.07	61 (569)	0.44	0.1
	4544 (1051)	0.24	0.25	4544 (671)	0.15	0.32	1051 (4544)	0.72	0.11	4544 (1051)	0.07	0.13
TAA	309 (76）	0.19	0.89	309 (76）	0.09	0.71	76 (309)	0.18	0.06	63 (309)	0.49	0.24
	3997 (903)	0.37	0.34	3997 (775)	0.15	0.95	903 (3997)	0.98	0.91	775 (3997)	0.1	0.12
WRA	289 (80)	0.95	0.69	289 (50)	0.07	0.31	80 (289)	0.11	0.21	50 (289)	0.17	0.92
	3898 (522)	0.29	0.71	3898 (570)	0.31	0.12	522 (3898)	0.07	0.15	570 (3898)	0.1	0.74
PBA	445 (86)	0.87	0.28	445 (41)	0.19	0.8	85 (445)	0.13	0.51	41 (445)	0.17	0.87
	3971 (247)	0.09	0.97	3971 (338)	0.11	0.68	247 (3971)	0.5	0.33	338 (3971)	0.39	0.21
DUA	443(101)	0.22	0.09	443 (75)	0.91	0.06	101 (443)	0.99	0.28	75 (443)	0.26	0.08
	4361(248)	0.07	0.39	4361 (413)	0.15	0.38	248 (4361)	0.34	0.22	413 (4361)	0.18	0.96

"Pb(As) bioaccessibility", 680(69) means 0.15 g soil spiked with 680 mg kg^{-1} Pb was mixed with 0.15 g soil spiked with 69 mg kg^{-1} As, etc; Bioaccessibility of Pb was compared with that of Pb in presence of As. "Pb(Cd) bioaccessibility", 680(51) means 0.15 g soil spiked with 680 mg kg^{-1} Pb was mixed with 0.15 g soil spiked with 51 mg kg^{-1} Cd, etc; Bioaccessibility of Pb was compared with that of Pb in presence of Cd. "As (Pb) bioaccessibility", 69(680) means 0.15 g soil spiked with 69 mg kg^{-1} As was mixed with 0.15 g soil spiked with 680 mg kg^{-1} Pb, etc; Bioaccessibility of As was compared with that of As in presence of Pb. "Cd(Pb) bioaccessibility", 51(680) means 0.15 g soil spiked with 51 mg kg^{-1} Cd was mixed with 0.15 g soil spiked with 680 mg kg^{-1} Pb, etc; Bioaccessibility of Cd was compared with that of Cd in presence of P

Appendix 4: Chapter 4 supplementary materials

Number of Tables: 1

Table S1 Total, bioaccessible concentrations and bioaccessibility of As, Cd and Pb in SRM 2710a and 2711a

SRM	metals/metalloid	Total concentration			Bioaccessible metals/metalloid (mg kg^{-1})		Bioaccessibility metals/metalloid (%)	
		Certified value (mg kg^{-1})	Measured value (mg kg^{-1})	Recovery (%)	Gastric phase	Intestinal phase	Gastric phase	Intestinal phase
2710a	As	1540±10	1594 ± 23	103±1.5	507±30	395±53	32±1.9	25±3.3
	Cd	12.3±0.3	11.4 ± 0.6	93±4.9	4.6±0.8	2.8±0.2	40±7.0[a]	25±1.8[a]
	Pb	5520±30	5579 ± 74	101±1.3	2571±55	578±119	46±1.0	10±2.1
2711a	As	107±5	90 ± 3	84±2.8	54±1.7[b]	42±5.4[b]	60±1.9[c]	47±6.0[c]
	Cd	54.1±0.5	50 ± 0.9	92±1.7	42±2.5[b]	10.8±1.1[b]	84±5.0	22±2.2
	Pb	1400±10	1340± 29	96±2.1	1021 ±42[b]	42 ±10[b]	76±3.1	3.1±0.7

[a, b. c] means data were in agreement with the following literature [a](Roussel et al., 2010), [b](Wragg et al., 2011), [c](Li et al., 2015b)

Data represents the results of six replicates. Please note that there are plenty of published bioaccessibility data for SRM 2710 and 2711 but since SRM 2710a and 2711a are renewal materials of SRM 2710 and 2711, limited published data are available for comparison. Especially for SRM 2710a, it was sampled from a different location from 2710

Appendix 5: Chapter 5 supplementary materials

Number of Tables: 2

Number of Figures: 1

Table S1 Total concentrations of magnesium (Mg), calcium (Ca), copper (Cu), iron (Fe), zinc (Zn), aluminium (Al) and manganese (Mn) in seven types of soils.

Total concentration in soils (g kg-1)	MIA	MGA	KBA	TAA	WRA	PBA	DUA
Mg	3.75	12.94	0.80	1.77	8.21	2.14	2.22
Ca	11.24	9.18	1.45	2.45	49.48	13.58	2.34
Cu	0.01	0.06	0.06	2.45	0.03	0.01	0.01
Fe	18.31	30.95	22.65	43.90	16.80	8.55	9.87
Zn	0.06	0.06	0.02	0.03	0.04	0.02	0.01
Al	33.07	26.99	11.13	56.64	28.31	12.01	7.98
Mn	0.07	0.50	0.06	0.27	0.37	0.09	0.17

Data represent mean of six replicates. Values varied by less than 3%.

Table S2 Concentrations of UBM-extracted As in DMEM-UBM solution in the presence of Cd or Pb

Soil code	As+Cd*		As+Pb*	
	Theoretical value (μM)	Measured value (μM)	Theoretical value (μM)	Measured value (μM)
MIA	11.97	12.88	10.09	11.37
	11.97	13.04		
MGA	2.11	2.19	2.05	2.05
	2.22	2.30		
KBA	13.54	13.86	5.83	6.06
	6.25	6.27	13.47	13.39
	15.72	14.99		
TAA	22.00	21.42	3.73	3.83
	8.90	9.02	19.45	19.44
	3.79	3.95		
	22.13	21.84		
	22.31	21.58		
WRA	7.19	7.28	6.04	6.58
	7.07	7.40		
	7.32	7.69		
	3.91	3.90		
PBA	3.90	3.72	3.42	3.41
	4.04	4.39		
DUA	3.85	3.81	3.68	3.62
	3.85	3.56		

"As+Cd", concentration of As in UBM-DMEM solution in the presence of Cd. "As+Pb", concentration of As in UBM-DMEM solution in the presence of Pb. "Theoretical value" means the calculated As concentration by dividing As concentration in UBM solution with dilution factor (10 in this study). "Measured value" means the measured As concentration in UBM-DMEM solution using ICPMS. Each data is mean of duplicate measurement. "*", students' *t*-test results demonstrate no significant difference was detected between "measured value" and "theory value" (p>0.05).

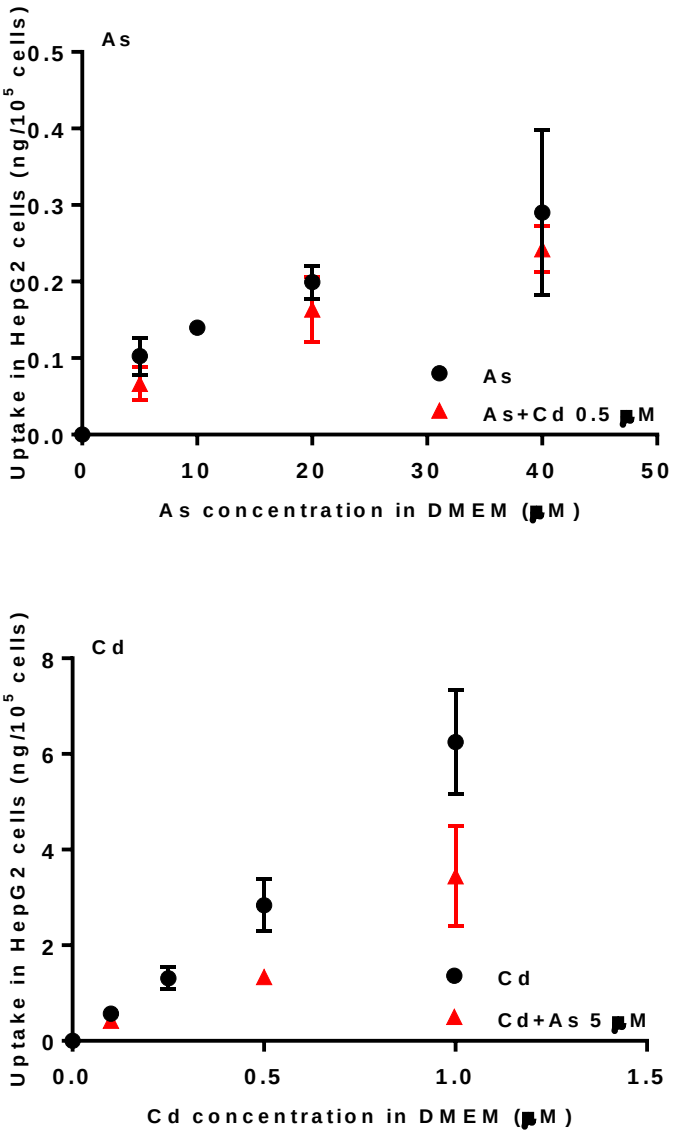


Figure S1 Interaction effects of pure solution As and Cd on respective uptake in HepG2 cells. Each data is the mean of at least triplicate measurement ±SD (standard deviation).

Appendix 6: Chapter 6 supplementary materials

Number of Table: 1

Number of Figure: 1

Table S1 Concentrations of UBM-extracted As, Cd and Pb in DMEM-UBM solution in the presence of PYR or B[a]P

Soil code	As+PYR		As+B[a]P		Cd+PYR		Cd+B[a]P		Pb+PYR		Pb+B[a]P	
	Theoretical value(μM)	Measured value(μM)	Theoretical value(μM)	Measured value(μM)	Theoretical value(μM)	Measured value(μM)	Theoretical value(μM)	Measured value(μM)	Theoretical value(μM)	Measured value(μM)	Theoretical value(μM)	Measured value(μM)
KBA	13.49	13.66	12.37	12.09	0.55	0.28	0.56	0.29	21.99	1.20	30.41	1.94
DUA	3.46	3.50	3.56	3.58	0.94	0.67	0.81	0.63	31.39	1.85	32.62	2.36

"Theoretical value" means the calculated As concentration by dividing As concentration in UBM solution with dilution factor (10 in this study). "Measured value" means the measured As concentration in UBM-DMEM solution using ICPMS. Similar explanation applies for "Cd+PYR", "Cd+B[a]P", "Pb+PYR", "Pb+B[a]P". Each data is mean of triplicate measurements. Students' t-test results demonstrated no significant difference was detected between 'measured value' and 'theory value' (p<0.05) for "As+ PYR" and "As+ B[a]P".

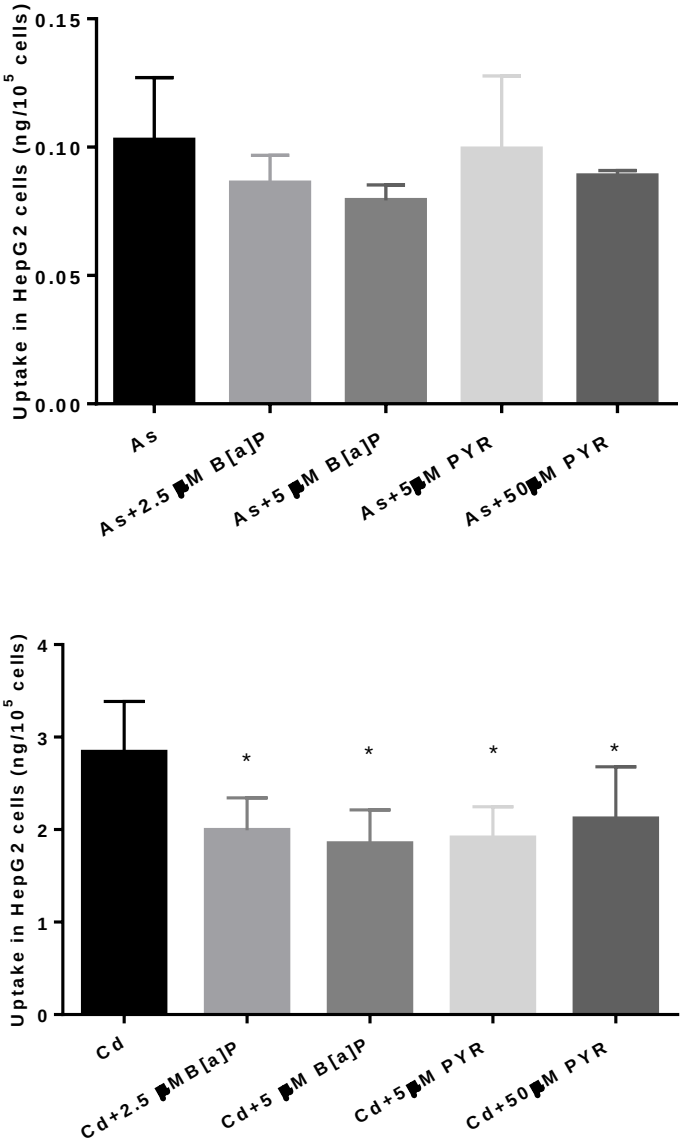


Figure S1 Effects of PYR and B[a]P on the uptake of pure solution As and Cd. Each data is mean ± standard deviation (SD). Bars with asterisk (*) mean data are significantly (p<0.05) different from that of control (Cd)